IMPROVING C4 PHOTOSYNTHETIC CHILLING TOLERANCE IN BIOENERGY CROPS: THE SEARCH FOR ELITE BREEDING MATERIALS

BY

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THESIS

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ABSTRACT

Increasing global energy demands and geopolitical instability among the world's major fossil fuel-producing countries have created an increased interest in alternative energy sources, particularly for liquid transportation fuels. Among these sources for alternative energy are biofuels, which entail the utilization of plant-based starch, sugar, oil, and potentially lignocellulosic biomass in the production of fuels. Due to their high productivity, C4 bioenergy feedstocks are among the top candidates for cultivation for biomass production, and crops such as sugarcane/energycane (Saccharum spp.), maize (*Zea mays*) , miscanthus (*Miscanthus spp*.), sorghum (*Sorghum bicolor*), and switchgrass (*Panicum virgatum*) have been suggested for the production of lignocellulosic biomass for bio-ethanol production. Under optimal conditions these C4 plants produce high biomass yields and have higher water and nitrogen use efficiencies than plants utilizing the C3 photosynthetic pathway, although these advantages are only seldom realized under suboptimal temperatures due to photosynthetic limitations at chilling temperatures (<20° C). The mechanistic limitation at chilling temperatures has been suggested to be caused by the chilling-induced lability of pyruvate phosphate dikinase (PPDK) and decrease in ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) enzyme contents, which directly limits the C4 cycle and indirectly leads to damage to the photosynthetic apparatus in the thylakoid membrane and generate harmful reactive oxygen species further downstream.

The bioenergy feedstock *Miscanthus x giganteus* is a leading candidate for biomass production in high latitudes of N. America, Europe, and Asia due to its high biomass yields and photosynthetic tolerance of mild chilling temperatures (15-20° C). However, *Miscanthus x giganteus* lacks tolerance of severe chilling conditions (0–12° C) and overnight frosts associated with early-season days in high latitudes. In chapter 2, two species from the *Miscanthus* genus are evaluated for their tolerance of severe chilling and overnight frost in the first comprehensive screening of a Siberian *Miscanthus sacchariflorus* germplasm collection. The objective here was to identify *Miscanthus* germplasm with superior tolerance of severe chilling and frost than those of *Miscanthus x giganteus*. One *Miscanthus sacchariflorus* accession was found to have higher photosynthetic chilling tolerance over a 15-day chilling period, and maintained higher net rate of carbon assimilation and quantum yield of photosystem II over the chilling period and on return to moderate temperature, while *Miscanthus x giganteus* maintained the highest rates of leaf elongation throughout the chilling treatment. Additionally, all *Miscanthus sacchariflorus* accessions showed comparable tolerance of overnight frost to that of *Miscanthus x giganteus*.

Energycane is a bioenergy feedstock candidate for biomass production in warm temperate and subtropical climates of the United States but photosynthetic limitations of the C4 cycle at suboptimal temperatures have limited the range at which it can be optimally cultivated, and the development of chilling-tolerant varieties is essential for extension of the growing season and mitigation of losses to biomass yields at higher latitudes. In chapter 3, I present an early-generation testing of the photosynthetic chilling tolerance of mild chilling temperatures in twenty six energycane hybrids generated from 10 crosses by the USDA-ARS basic breeding program in Houma, LA. The objective of this study is an early-generation screening of *Saccharum* spp. and inter-generic hybrids for *Saccharum* for superior photosynthetic chilling tolerance. The majority of energycane genotypes investigated here showed higher or comparable chilling tolerance than the chilling-intolerant *S. officinarum* 'LA Purple', while three energycane F1 hybrids (HB07-3452-4, HB07-3073-6, and HB07-3329-5) maintained the highest rates of leaf carbon assimilation over the chilling period and on return to moderate temperature.

The results presented in this thesis show that opportunity exists in wild germplasm material for the improvement of chilling tolerance in *Miscanthus x giganteus*, and that superior chilling tolerance can be generated in energycane through inter-specific and inter-generic crosses. In *Miscanthus* and energycane, severe chilling (10° C) and moderate chilling (15° C), respectively reduced the capacity for recovery under chilling conditions. Maintenance of photosynthetic capacity under chilling conditions and recovery at re-elevated temperatures are suggested here to be a function of regulation of specific enzymes involved in the C4 cycle, maintenance of thylakoid membrane proteins, light-energy dissipation strategies, generation of alternate electron sinks, and reactive oxygen scavenging.

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CHAPTER 1
GENERAL INTRODUCTION

Increasing global energy demands and geopolitical instability among the world's major fossil fuel-producing countries have created an increased interest in alternative energy sources, particularly for liquid transportation fuels. Among these sources for alternative energy are biofuels, which entail the utilization of plant-based starch, sugar, oil, and potentially lignocellulosic biomass in the production of fuels. Most importantly, within the context of global climate change, interest is also driven by the need to offset anthropogenic greenhouse gas emissions that are generated through the combustion of fossil fuels. Biofuels are viewed as an essential wedge in achieving net zero carbon emissions (Pacala and Socolow, 2004).

Sugarcane and maize are among the most commonly used plants for the production of bio-ethanol in the Americas (Nass *et al*., 2007), while cultivation of feedstocks such as miscanthus (*Miscanthus spp*.), sorghum (*S. bicolor*), switchgrass (*P. virgatum*), and energy cane (Saccharum spp.) have been suggested for the production of lignocellulosic biomass, which is can be converted into bio-ethanol. These species utilize the C4 photosynthetic pathway to rapidly and efficiently convert atmospheric CO_2 into vegetative biomass, sugars, and starches (Hatch *et al*., 1971).

As reviewed by Hatch *et al*. (1971) under most conditions the C4 photosynthetic pathway allows for the essential elimination of the costly process of photorespiration through modification of the leaf mesophyll and cell layer(s) of the bundle sheath around vascular bundles, known as Kranz anatomy (Sage, 2004; Hatch, 1987). Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), the enzyme that catalyzes the formation of 3-phosphoglycerate (3-PGA) molecules from ribulose-1,5-bisphosphate (RuBP) and CO_2 as the initial step of the Calvin-Benson-Bassham cycle, is sequestered to the stroma of chloroplasts of the bundle sheath cell layer(s), where CO_2 is released from malate within the C4 dicarboxylate photosynthetic cycle. This cycle essentially serves as a light-energy driven CO_2 concentrating mechanism (CCM), elevating CO_2 in the rubisco-containing bundle sheath to ca. 10x atmospheric levels (Hatch *et al*. 1971). Sugarcane, maize, sorghum, and Miscanthus are all of the NADP-ME-subtype of C4 photosynthesis; wherein atmospheric CO_2 is converted into HCO_3^- by carbonic anhydrase (CA) in the cytosol of mesophyll cells (Ludwig, 2011) and phosphoenolpyruvate carboxylase (PEPC) in the cytosol of these cells catalyzes the formation of oxaloacetate (OAA) from HCO_3^- and phosphoenolpyruvate (PEP). The OAA is then transported into chloroplasts of mesophyll cells, where malate dehydrogenase (MDH) catalyzes

the reduction OAA to malate by NADPH produced from the light-dependent reaction. The malate is then transported into chloroplasts of bundle sheath cells where it is decarboxylated by the NADP-malic enzyme (NADP-ME) to produce pyruvate, CO_2 for rubisco carboxylation, and NADPH is for the reduction of 3-PGA in Calvin-Benson-Bassham cycle (Hatch and Kagawa 1973). Finally, the pyruvate is transported back into chloroplasts of mesophyll cells where it is catalyzed into PEP through the addition of a phosphate group by pyruvate orthophosphate dikinase (PPDK) through the consumption of ATP (Hatch *et al*., 1971). Within the NADP-ME subtype of C4 photosynthesis rubisco and PPDK were found to be major flux controls of the entire pathway in a study involving antisense of key C4 photosynthetic enzymes in *Flaveria bidentis*, with flux coefficients of 0.7 for rubisco and 0.2-0.3 for PPDK under saturating light conditions (Furbank *et al*., 1997). It is now recognized that C4 sub-types are not restricted to one decarboxylation mechanism, and some decarboxylation occurs via PEP carboxykinase, while significant quantities of the C4 amino acid aspartate and C3 amino acid alanine may also be involved in transfer between the two photosynthetic tissues. The physiology and anatomical morphology associated with the C4 pathway ensure minimal rubisco oxygentation of RuBP and subsequent waste of potential ATP and NADPH associated with photorespiration (von Caemmerer and Furbank 2003).

Under optimum field conditions, especially at higher temperatures, the C4 photosynthetic pathway ensures high photosynthetic efficiency through the isolation of rubisco in the CCM of the bundle sheath cell layer (Hatch, 1987). However, at suboptimal temperatures under 20° C the mechanism ceases to function efficiently (Long, 1983), in part due to the chilling-induced lability of PPDK (Du *et al*. 1999) which decreases the availability of PEP as a substrate for PEPC carboxylation. This greatly reduces the plant's capacity to optimally assimilate carbon under saturating light (A_{sat}) as the CO_2-saturated photosynthetic rate (V_{pr}) is impaired by diminished PPDK activity (Du *et al*., 1999; Naidu and Long, 2004). In chilling-intolerant maize large reductions of PPDK and rubisco concentrations were observed in response to exposure to chilling conditions, and corresponded with reductions in A_{sat} and V_{pr} (Naidu *et al*., 2003; Naidu and Long, 2004, Wang *et al*., 2008; Long and Spence, 2013).

Another set of processes that get impaired under chilling conditions is the expression, degradation, and assembly of the D1 protein of PSII, which can lead to malfunction of PSII complexes (Grennan and Ort, 2007; Long and Spence, 2013; Spence *et al*, 2014). Although PSII complexes only appear in the mesophyll of NADP-ME subtype C4 species, the reductive potential from the light-dependent reaction is transported into bundle sheath in the form of malate, from which NADPH for the Calvin-Benson-Bassham cycle is generated by NADP-ME

decarboxylation of malate. The reduction in overall PSII activity does not generally limit photosynthesis under high light conditions where there is sufficient NADPH, however, it may impair the maximum quantum yield of CO_2 uptake under light-limiting conditions (Glowacka *et al*., 2015a). Under these conditions electron flux from photosystem I (PSI) onto dioxygen can cause the formation of superoxide, hydrogen, peroxide, and hydroxyl radials that can be extremely damaging to thylakoid membrane proteins (Ort and Baker, 2002). To reduce photodamage plants have been shown to produce zeaxanthin via the xanthaphyll cycle to dissipate excess light as heat (Farage *et al*., 2012), initiate the water-water cycle (Ort and Baker, 2002), and express reactive oxygen-scavenging enzymes (Jahnke *et al*., 1991; Fryer *et al.*, 1998). Accordingly, chilling-intolerant C4 species may experience sizeable yield losses and reductions of photosynthetic capacity if exposed to chilling temperatures for prolonged periods of time (Ercoli, *et al*., 2004). Photosynthesis in crops such as maize, sorghum, and sugarcane crashes after prolonged periods of chilling, these plants cannot reap the benefits of the C4 mechanism (Nie *et al*. 1992; Du *et al*. 1999; Ercoli *et al*. 2004; Dohleman and Long 2009; Long and Spence, 2013). Indeed, the evolution of C4 photosynthesis has kept these chilling-intolerant species at low latitudes and altitudes (Sage *et al*. 2011).

The *Miscanthus* and *Spartina* genera appear unique, with members the *Miscanthus* genus forming dominant components of the vegetation in areas as far north as Sapporo Island in Japan for *M. sinensis* (*Msi*) and Southeastern Siberia for *M. sacchariflorus* (*Msa*) (Sacks *et al*., 2013), and members of the *Spartina* genus thriving in high latitudes in Western Europe, Northern Europe, and North America (Long *et al*., 1975; Long and Spence, 2013). *Miscanthus x giganteus* (*Mxg*), the highly productive sterile triploid hybrid between *Msi* and *Msa*, has been noted as an exceptional chilling-tolerant bioenergy crop due to its high yield potential and photosynthetic chilling tolerance (Beale and Long, 1995; Beale *et al*., 1996; Dohleman and Long, 2009; Purdy *et al*., 2013). Studies have shown that *Mxg* has high-yield potential in temperate areas, with yields reaching as high as 30 Mg h^{-1} in Midwestern USA and 20 Mg h^{-1} in England (Heaton *et al*, 2008; Beale and Long 1995). In comparison to maize, *Mxg* has been shown to shown to produce photosynthetically competent leaves earlier and maintain them later, allowing it to be almost 60% more productive (Dohleman and Long, 2009). One key aspect of *Mxg* chilling tolerance appears to be its capacity to up-regulate the content of PPDK and also its ability to maintain its Rubisco content when exposed to mild chilling temperatures (>14° C) (Wang *et al*., 2008; Naidu *et al*., 2003, Naidu and Long, 2004; Long and Spence 2013). Additionally, in a recent microarray study, it has been shown that genes for key plastid proteins that are down-regulated in maize during chilling are up-regulated in *Mxg* (Spence *et al*. 2014).

Mxg shows a strong up-regulation of zeaxanthin content at severe chilling temperatures (<12° C), which protect it against photoinhibitory damage to photosystem II (Farage *et al*., 2006), although the rates of carbon assimilation are not recovered at these temperatures (Purdy *et al*., 2013; Friesen at al., 2014; Glowacka, *et al*., 2014). While *Mxg* shows exceptional chilling tolerance of photosynthesis in comparison to other C4 species, further improvement would allow cultivation into colder climates and extend the growing season in its current areas of cultivation. Glowacka *et al*. (2015a) recently identified genotypes of *Msa* that showed significantly greater chilling tolerance of photosynthesis than *Mxg*. However, these genotypes were collected on Honshu, which suggests that greater chilling tolerance might yet be found by examining genotypes native to more northerly locations in E. Asia.

Sugarcane is a notoriously chilling-intolerant C4 crop, even by comparison to maize, and has been shown to be highly susceptible to chilling damage and reductions in productivity (Faris, 1926; Verret and Das, 1927; Grantz, 1989; Campbell *et al*., 1998; D'Hont *et al*., 2008; Inman- Bamber *et al*., 2010; Inman-Barber *et al*., 2014; Friesen *et al*., 2014). This currently limits the crop in mainland USA to regions fringing the Gulf coast, and southern Florida. As such, in recent years interest has grown in making the crop more chilling tolerant, including hybridization with chilling-tolerant lines of *Msa* and *Msi* (Glowacka *et al*. 2014; Glowacka *et al*. 2015a; Glowacka *et al*. 2015b), and chilling-tolerant *S. spontaneum* (Khan *et al*., 2013; Daniel and Roach, 1987). *S. spontaneum* is known for its superior chilling/freezing tolerance within the *Saccharum* genus (Brandes, 1940).

The research in this thesis represents pre-breeding efforts as well as phenotypic evaluations to identify potential parental material for increasing chilling tolerance of photosynthesis in the bioenergy crops energycane and *Miscanthus*. The first study (Chapter 2) tests the hypothesis that *Msa* collected in SE Siberia will show improved chilling and frost tolerance relative to *Mxg*, with the aim of identifying germplasm for breeding improved *Mxg*. The second study (Chapter 3) tests the hypothesis that energycane hybrids produced from wide inter- and intra-genus crosses within the *Poaceae* will exhibit greater chilling tolerance than the historically-cultivated *S. officinarum* 'LA Purple', with the aim of indentifying lines with superior chilling tolerance for future breeding efforts.

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CHAPTER 2

SCREENING OF A NOVEL SIBERIAN *MISCANTHUS SACCHARIFLORUS* GERMPLASM FOR SUPERIOR PHOTOSYNTHETIC CHILLING AND FROST TOLERANCE

ABSTRACT

The bioenergy feedstock *Miscanthus x giganteus* (*Mxg*) is a leading candidate for biomass production in high latitudes of N. America, Europe, and Asia due to its high biomass yields and photosynthetic tolerance of mild chilling temperatures (15-20° C). However, *Mxg* lacks tolerance of severe chilling conditions (0–12° C) and overnight frosts associated with early-season days in high latitudes. The identification of germplasm material with superior chilling/freezing tolerance is crucial for breeding *Miscanthus* that is competitive with candidate C3 crops in these climates. The objective here was to identify *Miscanthus* germplasm with superior chilling tolerance and frost tolerance capacities to those of *Mxg*. This is the first in-depth evaluation of photosynthetic chilling tolerance in the *Miscanthus sacchariflorus* germplasm collection that was collected in SE Siberia in late 2012. In this experiment the chilling tolerance of 91 Siberian *Miscanthus sacchariflorus* (*Msa*) accessions was evaluated under field conditions following a 13-day chilling period (13.1-15.9° C) by fluorescence measurements of the maximum efficiency of PSII (F_v/F_m). The chilling/freezing tolerance of seven of the top-performing *Msa* accessions as well as *Mxg* were further investigated under controlled environment conditions through leaf gas exchange, fluorescence, and leaf elongation. The chilling treatment involved the transfer of plants grown under warm temperatures 25°/20° C day/night to 10°/5° C day/night temperature conditions for 15 days, followed by a one day of re-elevation of temperatures to 25° C. The frost treatment involved the transfer of plants grown under warm temperatures 25°/20° C day/night to 10°/5° C day/night temperature conditions for 48 hours, during which they were exposed to a three-hour exposure to -2° C temperatures overnight, followed by a one day of re-elevation of temperatures to 25° C. Two *Msa* accessions were found to have higher photosynthetic chilling tolerance than *Mxg* over the 15-day chilling period, and maintained higher net rate of carbon assimilation and quantum yield of photosystem II over the chilling period as well as the re-elevation of temperatures, while *Mxg* maintained the highest rates of leaf elongation throughout the chilling treatment. Conversely, *Mxg* maintained the highest photosynthetic carbon assimilation following the frost treatment, while one *Miscanthus sacchariflorus* showed a greater quantum yield than *Mxg* following the frost treatment. In conclusion, two Siberian *Msa* accessions were identified to

have superior chilling tolerance than that of exceptionally chilling-tolerant *Mxg*, one of which also holds a superior frost tolerance of the photosynthetic apparatus in the thylakoid membrane.

INTRODUCTION

Miscanthus x giganteus (*Mxg*), the hybrid between *M. sacchariflorus* (*Msa*) and *M. sinensis* (*Msi*) is widely considered for cultivation as a second-generation lignocellulosic bioenergy crop in temperate climates due to its high biomass yields and photosynthetic chilling-tolerance (Beale and Long, 1995; Beale *et al*., 1996; Dohleman and Long, 2009; Heaton *et al*., 2010; Jones, 2011; Long and Spence, 2013). *Mxg*, as a C4 crop, is highly productive at temperate climates, producing biomass yields of 20 Mg h^{-1} in England and yields of 30 Mg h^{-1} in the US "corn belt" (Beale and Long, 1995; Heaton *et al*.,2008).

Species that evolved the C4 photosynthetic pathway have been shown to have high efficiency in the photosynthetic assimilation of atmospheric CO_2 (Bauwe, 2011), water-use efficiency, and nitrogen-use efficiency under optimal field conditions (Sage, *et al*., 1987; reviewed: Ghannoum *et al*. 2011). However, at suboptimal temperatures under 20° C the mechanism ceases to function efficiently (Long, 1983), in part due to the chilling-induced lability of pyruvate orthophosphate dikinase (PPDK) (Du *et al*. 1999a; Du *et al*. 1999b), which decreases the availability of phosphoenolpyruvate (PEP) as a substrate for PEPC carboxylation, and C4 crops that lack photosynthetic chilling tolerance are highly susceptible to damage by prolonged periods of suboptimal temperatures (Sage *et al*., 2011; Long and Spence, 2013). In NADP-ME subtype C4 crops, the drop in PEP availability due to decreases in PPDK activity causes decreases in photosynthetic efficiency under saturating light (A_{sat}) as the CO_2-saturated photosynthetic rate (V_{pr}) is impaired by diminished PPDK activity (Du *et al*., 1999; Naidu *et al*., 2003; Naidu and Long, 2004; Wang *et al*., 2008). Another set of processes that are impaired under chilling conditions is the expression, degradation, and assembly of the D1 protein of PSII, which can lead to malfunction of PSII complexes (Grennan and Ort, 2007; Long and Spence, 2013; Spence *et al*., 2014). Although PSII complexes only appear in the mesophyll of C4 species, the reductive potential from the light-dependent reaction is transported into bundle sheath in the form of malate, from which NADPH for the Calvin-Benson-Bassham cycle is generated by NADP-ME decarboxylation. The reduction in overall PSII activity does not generally limit photosynthesis under high light conditions where there is sufficient NADPH, however, it does impair the maximum quantum yield of CO_2 uptake under light-limiting conditions (Glowacka *et al*., 2015a).

Due to their exceptional photosynthetic chilling tolerance capacities and freezing-tolerance during dormancy, members of the *Miscanthus* genus out-perform most other C4 crops under the chilling conditions of early and late season (Farrell *et al*., 2006; Naidu *et al*., 2003; Wang *et al*., 2008; Clifton-Brown and Lewandowski, 2000). Accordingly, *Mxg* retains relatively high net leaf CO_2 assimilation rates (A) at temperatures below 20° C and produces a functional canopy earlier in the growing season in comparison to other widely-cultivated C4 crops in temperate climate areas (Dohleman and Long, 2009; Beale *et al*., 1996; Beale and Long, 1995). A study comparing the responses of chilling-susceptible maize and chilling tolerant *Mxg* showed prolonged exposure to mild chilling temperatures (>14° C) caused the decrease of Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) and PPDK protein contents of maize by 40% and 70%, respectively, while *Mxg* circumnavigated the lability of PPDK with up-regulation of PPDK protein contents by as much as 200% and full recovery of rubisco protein contents over a two-week period (Wang *et al*., 2008; Long and Spence, 2013). Additionally, in a recent microarray study, it has been shown that genes for key plastid proteins that are down-regulated in maize during chilling are up-regulated in *Mxg* in response to mild chilling temperatures (Spence *et al*. 2014). However, to date there are no studies depicting PPDK, rubisco, or plastid gene expression/protein regulation in *Mxg* under severe chilling temperatures (<12° C). The accumulation of zeaxanthin in *Mxg* at 10° C but not at 15° C for non-photochemical quenching of excess light-energy (Farage *et al*., 2006) suggests a lack of *Mxg* tolerance of severe chilling temperatures such as that observed in maize in response to mild chilling temperatures, and may imply an upregulation of enzymes involved in the water-water cycle and enzymes involved in oxygen-scavenging (Jahnke *et al*., 1991; Fryer *et al*., 1998; Ort and Baker, 2002). Whereas Wang *et al*. (2008) observed a full recovery of photosynthetic rates in *Mxg* under mild chilling conditions, Glowacka *et al*. (2014) and Friesen *et al*. (2014) showed that *Mxg* lacks the capacity for recovery of photosynthetic rates over prolonged periods of exposure to severe chilling temperatures.

The exceptional chilling tolerance of *Miscanthus* allows it to develop photosynthetically competent leaves and begin assimilating carbon early in the season (Dohleman and Long, 2009), which creates a vulnerability in *Miscanthus* to early-season frost damage (Glowacka *et al*., 2015a) and damage from cold temperatures below 10 °C (Farage *et al*., 2006). At these low temperatures even the accumulation of rubisco, PPDK, and zeaxanthin pigments is not be sufficient to avoid damage under saturating light conditions (Kubien *et al*., 2003; Sage *et al*., 2011). Formation of ice crystals in intercellular spaces can potentially result in disruption of cell tissue and membranes by dehydration (Uemura *et al*. 1995; Pearce, 1999), and freezing

temperatures have been shown to cause mild to severe yield losses in many crops (Xin and Browse 2000).

At present, all *Mxg* cultivated for biomass production is clonally derived from material propagated from a single hybrid that was originally collected in Honshu, Japan (Greef and Deuter, 1993). This sterile triploid is believed to be the result of a natural hybridization between a tetraploid *Msa* and diploid *Msi* (Linde-Laursen, 1993; Hodkinson *et al*., 2002). However, the monoculture cultivation of a single genotype leaves large biomass -production fields susceptible to potential insect, fungus, and disease infestations (Ahonsi *et al*.,2010; Prasifka *et al*., 2009; Bradshaw *et al*.,2010), and does not allow for optimal growth across a wide variety of field conditions and climates. In recent years, large efforts have been made in the improvement of *Mxg* yields, and new *Mxg* varieties have been hybridized from *Msa* and *Msi* collected from cold locations in Eastern Asia to improve chilling tolerance and yield stability under chilling conditions (Deuter, 2000; Chae *et al*., 2013). Additionally, the search for superior parental lines for the hybridization of novel *Mxg* genotypes with superior photosynthetic chilling tolerance have produced mixed results (Purdy, *et al*., 2013; Friesen, *et al*., 2014; Glowacka, *et al*., 2014; Glowacka, *et al*., 2015a). Glowacka *et al*. (2015a) recently identified genotypes of *Msa* that showed significantly greater chilling tolerance of photosynthesis than *Mxg*. These genotypes were collected on Honshu, which suggests that greater chilling tolerance might yet be found by examining genotypes native to more northerly locations in E. Asia. Indeed, to be maximally useful breeding for increased chilling tolerance in *Mxg* will necessitate maintenance of high photosynthetic capacities as well as resistance to frost damage.

In this study, the photosynthetic tolerance of severe chilling temperatures over a 15-day period (10° /5° C day/night) and overnight frost-tolerance (-2° C) of an *Msa* germplasm collected from SE Siberia was evaluated against that of the exceptionally chilling-tolerant *Mxg*. In June of 2014, during the last 3 days of a 13-day chilling period in central Denmark 91 Siberian *Msa* accessions were screened under field conditions for maintenance of maximum efficiency of PSII (F_v/F_m) in order to find accessions with minimal damage to PSII. Seven accessions that showed the highest F_v/F_m values were cloned and transferred into controlled environment chambers to further investigate capacity for growth and photosynthetic chilling tolerance under severe chilling conditions (10° C day / 5° C night) and recovery from an overnight frost (-2° C). Here we test the hypotheses that material collected from the high-latitudes of Siberia will exhibit (1) greater photosynthetic tolerance of severe chilling temperatures than the commonly-cultivated *Mxg* both in terms of maintenance of vegetative growth and light-saturated photosynthesis, and (2)

greater leaf freezing tolerance than *Mxg* in terms of maintenance of photosynthetic capacity following an overnight freezing event.

MATERIALS AND METHODS

Plant material in the field

In the fall of 2012, 222 local accessions of *Miscanthus* were collected as rhizomes and seeds from regions across Southeastern continental Siberia, Russky Island, and Sakhalin Island. This was a USDA-funded international collaboration involving between Dr. Erik Sacks from the University of Illinois, Dr. Doug Johnson from Utah State University, and Dr. Nikolay Dzyubenko, Dr. Elena Dzyubenko, Dr. Larisa Bagmet, Andrey and Dr. Sabitov from the Vavilov Research Institute. Overall, 202 accessions of *Msa* and 20 accessions of *Msi* were collected. These accessions were grown under greenhouse conditions for ten months of quarantine at the Foulum filed station at the University of Aarhus. After the quarantine period they were transferred to the field where two clones of each accession were planted 150 cm apart in late summer of 2013. *Mxg* was also propagated from rhizome in a separate neighboring plot in late summer of 2013. These fields are located at the Aarhus University Research Center at Foulum in Central Jutland, Denmark (56° 30' N, 9° 35' E), the soil is a sandy loam (typic Fragiudalf; USDA soil taxonomy) (Larsen *et al*., 2014; Glowacka *et al*., 2015a).

Field measurements of F_v/F_m

In late June 2014 between June 19th and July 2nd, a 13-day chilling period occurred at this field site where average air temperatures did not exceed 15° C for eleven out of the thirteen days, with average day-time temperatures averages raging between 13.13-15.90° C during this period (Fig. 1). The 91 Siberian *Msa* accessions showing the least amount of damage, based on visual evaluation of leaf chlorosis, were chosen for assessment of maximum efficiency of PSII (F_v/F_m) *in situ* by chlorophyll fluorescence, *Mxg* growing in an adjacent plot was included in measurements (Table 1). Each accession was represented by two separate shoots in each of two replicate plantings in the field. The ratio of variable to maximal leaf chlorophyll fluorescence (F_v/F_m) was measured by applying a saturating rectangular pulse of light (state PPFD) using a pulse amplitude-modulated fluorometer (LI-6400-40; LI-COR, Lincoln, NE, USA) protocol followed the procedures of Glowacka *et al*. (2015a). F_v/F_m was obtained by measuring the minimum chlorophyll fluorescence (F_0) and maximum chlorophyll fluorescence (F_m) during the saturating pulse; where $F_v/F_m = (F_m-F_0)/F_m$ (Maxwell and Johnson, 2000). Measurements of

F_v/F_m were performed overnight over three consecutive nights between June 29th and July 1st under complete darkness 0.5 h after external conditions reached 0 µmol photons m^{-2} s^{-1}. We measured horizontal portions of the lamina in the most recent fully expanded set of leaves (denoted by an emergent ligule) that are not shaded during the day by other leaves. On the first night of measurements 39 *Msa* accessions were measured along with *Mxg*, on the second night of measurements 42 *Msa* accessions were measured along with *Mxg*, and on the third night of measurement ten of the top-performing *Msa* accessions from each of the two former nights were re-measured along with 11 additional *Msa* accessions and *Mxg* to ensure homogeneous measurement conditions.

Plant material in the greenhouse

On transfer of the collection from USDA quarantine to controlled environment glasshouses at the University of Illinois, 7 Siberian *Msa* accessions were chosen for their apparent chilling tolerance, based on their field performance in Denmark and availability of sufficient clonal material for further investigation. *Mxg* was included as a positive chilling-tolerant control. Clonal divisions of these *Miscanthus* accessions were grown in 1.6-liter round pots (15 cm Euro pot #EU150T5; Euro System, United Kingdom) containing a peat/bark/perlite- based growing medium (Metro-Mix 900; Sun Gro Horticulture, Agawam, MA, USA). After cloning by rhizome propagation, a slow release fertilizer was added according to the manufacturer's instructions (Osmocote Pro, 8–9 mo 19-5-8 Minors; Everris NA, Inc., Dublin, OH, USA). Plants were grown in a controlled-environment greenhouse at the University of Illinois, Champaign-Urbana at ~25° C under a 14 h light/12 h dark cycle for 53 days prior to transfer to controlled-environment growth cabinets (Fig. 2 A-B). Soil moisture content was maintained to field capacity by a daily watering regimen.

15-day severe chilling treatment

Leaf elongation, gas exchange, and chlorophyll fluorescence were evaluated for all 7 *Msa* accessions and *Mxg* under controlled environmental conditions. Four potted plants of each accession were transferred from the greenhouse to a controlled-environment chamber (PGC20 Growth Chamber; Conviron, Winnipeg, Manitoba, Canada) with a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1}, 25 °C daytime/20 °C nighttime temperature, and relative humidity of 75%. After 23 days of acclimation to the growth chamber, leaf carbon uptake rates and leaf elongation rates were analyzed for four consecutive days to evaluate the health of each plant. Plants which failed to grow, showed marked drops in carbon assimilation rates, showed

necrosis, or chlorosis were removed from the study. We then included 1 day of control measurements under warm conditions (25 ° /20 °C day/night), after which the temperature in the growth chamber was lowered to severe chilling temperatures (10°/5 °C day/night) for 15 d, and then returned to warm temperatures for 1 d (Fig. 2A). This 15-day period was chosen to emulate the type of chilling that developing plants might experience during vegetative growth in spring, and follows the bioassay developed by Wang *et al*. (2008) and used by Glowacka *et al*. (2014) and Glowacka *et al*. (2015a). Aside from temperature, all other environmental conditions were unchanged. To avoid the confounding effects of heterogeneous environmental conditions within the growth cabinet, each plant within the growth cabinet was rotated every two days according to a fully randomized design.

Leaf elongation rates were measured each day for 5 concurrent days under warm conditions during the daytime on the newest non-ligulated leaf on one stem per pot, and leaf elongation rates were measured in the same fashion on days 1, 2, 5, 6, 8, 10, 12, and 15 of the chilling treatment. To ensure a static base from which to measure leaf length, 12.5 cm wooden stakes were inserted into the soil of each pot and the distance from the top of the stake to the leaf tip was measured using a ruler. For each plant, the rate of leaf growth (cm d^{-1}) was the difference in leaf height from the last measurement day divided by the number of days that have passed between the days (i.e. Δ leaf length/# days).

Leaf photosynthetic gas exchange was measured *in situ* on the most recent fully expanded leaf, as indicated by ligule emergence, with an open gas exchange system incorporating CO_2 and water vapor analyzers (LI-6400; LI-COR, Lincoln, NE, USA). Leaf photosynthetic gas exchange was measured on the 4 days prior to the induction of the chilling treatment, and then measured on days 1, 2, 5, 6, 8, 10, 12, and 15 of the chilling treatment. All measurements were taken between 3-8 h into the day portion of the photoperiod on light-adapted leaves by measuring precisely the same portion of the leaf on each measurement day. The leaf was enclosed in a controlled-environment cuvette with tracked temperature, humidity, and light. Measurements were conducted under ambient air (21% O_2) at 400 ppm CO_2 concentration, 2000 μmol m^{-2} s^{-1} photon flux, 75% relative humidity, and leaf temperature was controlled at the growth temperature for each treatment. Light-emitting diodes were used for actinic light (90% red light, 630 nm; 10% blue light, 470 nm). The net CO_2 uptake rate per leaf area (A), stomatal conductance to water vapor (g_s), and intercellular CO_2 concentration (C_i) were calculated, as described in Bernacchi *et al*. (2003). Chlorophyll pulse amplitude fluorescence was measured simultaneously with leaf photosynthetic gas exchange by a fluorometer positioned in the cuvette lid (LI-6400-40; LI-COR, Lincoln, NE, USA). A multiphase

flash protocol was used to maximize fluorescence emissions. The quantum yield of photosystem II under saturating light (Φ_{PSII}) was calculated as described by Maxwell and Johnson (2000). The cuvette was attached to the leaf for 10-20 minutes prior to measurement in order to achieve steady-state photosynthesis and stomatal response.

Overnight freezing treatment

Gas exchange and chlorophyll fluorescence were evaluated for all 7 Siberian *Msa* accessions and *Mxg* under controlled environmental conditions. Four potted plants of each accession were transferred from the greenhouse to two controlled-environment chambers (E15 Growth Chamber; Conviron, Winnipeg, Manitoba, Canada) with a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1}, 25 °C daytime/20 °C nighttime, and relative humidity of 75%. After 11 days of acclimation to the growth chamber, leaf carbon uptake rates were analyzed for four concurrent days to evaluate the health of each plant, plants that exhibited unreasonable fluctuations in carbon assimilation were removed from the study. As shown in Fig. 2B, an additional day of measurements (day 0 of the freezing treatment) at 25 °C daytime/20 °C nighttime (warm) temperatures was included, after which the temperature in the growth chambers was lowered to 10 °C daytime, followed by 5 °C nighttime, and plants were transferred to a dark -2° C incubator (Model 818 Incubator; Thermo Scientific, Marietta, OH, USA) for three hours half-way through the dark period. The plants were then returned to 5° C for the remainder of the dark photoperiod, followed by one day of temperature settings at 10 °C daytime/20 °C nighttime, and a day of 25 °C daytime temperatures. Half of the shelves inside the incubator were lined with frozen pots of damp soil ahead of time to ensure retention of freezing temperatures upon placement of plants in the incubator. The overnight freezing period was chosen to emulate the type of frost that young shoots might experience during early vegetative growth in spring. Aside from temperature, all other environmental conditions were unchanged. To avoid the confounding effects of heterogeneous environmental conditions within the growth cabinet, each plant within the growth cabinet was randomly rotated every day. Chlorophyll pulse amplitude fluorescence and leaf photosynthetic gas exchange were measured *in situ* everyday with the same protocol as described above for the 15-day chilling treatment.

Data Analysis

Statistical analyses were performed with SAS v. 9.3 (SAS Institute, Cary, NC, USA). All statistical analyses were performed at alpha 0.1. The fixed main effect of accession on F_v/F_m was determined by a one-way analysis of variance (ANOVA), post-hoc Dunnett's mean

separation tests were used to determine significant differences from *Mxg* per each measurement day. The fixed main effect of accession and time in the chilling/freezing treatment on all measures (leaf elongation rate, A, g_s, C_i/C_a, Φ_{PSII}, and relative changes of said variables) were determined by a two-way repeated-measures ANOVA in PROC MIXED with an autoregressive covariance structure. In the presence of a significant main effect of accession on any variable, post-hoc Dunnett's mean separation tests were used to determine significant differences from *Mxg* per each measurement day.

RESULTS

Maximum efficiency of photosystem II (F_v/F_m)

Ninety two accessions were identified in the field that showed minimal chlorosis and overall damage during the 13-day chilling event in June 2014 in Foulum, Denmark (Table 1). Thirty eight, thirty one, and twenty one accessions showed significantly higher Fv/Fm values than *Mxg* on the first, second, and third nights of measurements, respectively (Fig. 3). On the third night of measurement, ten of the best performing accessions from each of the previous nights of measurement were re-measured along with 11 additional previously un-measured accessions. F_v/F_m values ranged from 0.772 to 0.619 on the third night of measurement, with *Msa* accession RU2012-091 having the highest F_v/F_m average of 0.743 and *Mxg* having the lowest F_v/F_m average of 0.632 (Fig. 3C).

Fifteen-day chilling treatment – leaf elongation, photosynthetic gas exchange & chlorophyll fluorescence

Mxg, the chilling-tolerant positive control showed the lowest overall reduction in leaf elongation rate with a 88.9% reduction from 3.47 cm d^{-1} under warm conditions (25° / 20° C day/night) to 0.38 cm d^{-1} at chilling conditions (10°/ 5° C day/night), as shown in Fig. 4. Of the 7 *Msa* accessions analyzed, five accessions (RU2012-069, RU2012-114, RU2012-112, RU2012-121, and RU2012-091) showed significantly higher reductions in leaf elongation from *Mxg* under chilling temperatures with rates of decrease of leaf elongation at chilling conditions ranging between 95.8-96.8% in these accessions ($P \leq 0.1$; Fig. 4C).

Over the 15-day chilling treatment, "miscanthus accession" and "days of chilling" had significant effects on all gas exchange measurements (A, g_s, C_i/C_a) and ϕ_{PSII} ($p<0.0001$), while the day-by-accession interaction was significant for A, C_i/C_a, and ϕ_{PSII} ($p<0.001$, $p<0.1$, and $p<0.1$, respectively). Overall, *Msa* accession RU2012-114 maintained higher rates of net CO_2

uptake rate per leaf area (A) than *Mxg* following the 15-day chilling treatment (Fig. 5H), with 46.6 μmol m^{-2} s^{-1} at warm conditions, 11.4 μmol m^{-2} s^{-1} (75.6% decrease) after 15 days of chilling, and 22.8 μmol m^{-2} s^{-1} (51.0% decrease) upon return to 25° C following the 15-day chilling treatment; compared to *Mxg*, which maintained average rates of 28.6 μmol m^{-2} s^{-1}, 4.94 μmol m^{-2} s^{-1} (82.7% decrease), and 14.3 μmol m^{-2} s^{-1} (50.0% decrease), respectively. As shown in Fig. 6H, *Msa* RU2012-114 also had significantly higher recovery rates of ϕ_{PSII} after the re-elevation of temperature following the 15-day chilling period, having only lost 32.9% efficiency in the quantum yield of photosystem II, compared to a 55.8% lost by *Mxg*. *Msa* accession RU2012-073 maintained significantly higher stomatal conductance of water vapor (g_s, Fig. 7D-E) than *Mxg* throughout the 15-day chilling treatment with a 50.8% reduction of stomatal conductance compared with the 88.6% reduction in *Mxg* g_s on the 15th day of chilling. *Msa* accessions RU2012-073 and RU2012-121 showed a significantly greater increases in the ratio of intercellular-to-atmospheric CO_2 (C_i/C_a) than *Mxg* on the 15th day of the chilling treatment (88.8% and 68.0%, respectively, Fig. 8E), compared with a mere 27.7% increase in *Mxg* and 8.34% increase in *Msa* RU2012-114. Relative changes in A, ϕ_{PSII}, g_s, and C_i/C_a in the top-perfuming accessions and worst performing accessions are depicted in Fig. 9.

Overnight freezing treatment–photosynthetic gas exchange & chlorophyll fluorescence
There were no significant differences in either the reduction of A or in the reduction of ϕ_{PSII} between *Mxg* and any of the *Msa* accessions. All *Msa* accessions and *Mxg* showed similar reductions of A and ϕ_{PSII} following the overnight freezing treatment (Fig. 10F).

DISCUSSION

In this experiment we found one Siberian *Msa* accession with superior C4 photosynthetic chilling tolerance to that of *Mxg*, four *Msa* accessions that matched *Mxg's* chilling tolerance, and we found that all *Msa* accessions studied had comparable freezing tolerance to those of *Mxg*. This is the first in-depth evaluation of photosynthetic tolerance to severe chilling temperatures and overnight frost in the *Msa* germplasm collection that was collected in SE Siberia in late 2012. Here the chilling tolerance of 91 Siberian *Msa* accessions was evaluated in the field, while the severe chilling/freezing tolerance capacities of 7 accessions was studied in-depth under controlled environment conditions in comparison to *Mxg*.

Of the accessions evaluated in this study, *Msa* accession RU2012-114 maintained higher F_v/F_m values than *Mxg* during the 13-day chilling period in the field in Foulum, Denmark (Fig. 3C), and under controlled conditions it maintained the highest photosynthetic rates at 25°

C before the initiation of the chilling treatment, highest photosynthetic rates throughout the 15-day chilling treatment, and highest photosynthetic rates at re-elevation of temperatures to 25° C (Fig. 5). This accession also maintained high ϕ_{PSII} throughout the experiment and maintained higher ϕ_{PSII} values than *Mxg* during the first day of exposure to 10° C and at re-elevation of temperatures to 25° C (Fig. 6). To date, this is the first report of a *Miscanthus* accession that out-performed the exceptionally chilling-tolerant *Mxg* at severe chilling temperatures. In a previous study Glowacka *et al.* (2015a) found two *Msa* accessions of Japanese origin that out-performed *Mxg* under moderate chilling conditions, although the tolerance of severe chilling temperatures of these accessions is not currently known.

In this study we also identified four accessions whose tolerance of severe chilling temperatures matched that of *Mxg*. The rates of decrease of A and ϕ_{PSII} in *Msa* accessions RU2012-069, RU2012-073, RU2012-112, and RU2012-121 did not significantly differ from those of *Mxg* during the 15-day chilling treatment. This adds to the growing body of works showing that the exceptional photosynthetic chilling tolerance of *Mxg* can be matched even at severe chilling temperatures. Glowacka *et al.* (2014) and Glowacka *et al*. (2015b) reported one *Msa* accession and two 'miscane' hybrids whose reduction in A did not significantly differ from *Mxg* in response to exposure to 11 days of severe chilling temperatures, while Friesen *et al.* (2014) found two *Miscanthus* hybrids and one energycane genotype with comparable photosynthetic chilling tolerance to that of *Mxg* after 7 days of severe chilling. It is important to mention that the aforementioned *Msa* accession RU2012-112 was chosen for having the worst values of F_v/F_m during the 13-day chilling period in the field but it maintained rates of A comparable to *Mxg* and *Msa* RU2012-114 after 15 days of chilling and experienced the lowest reduction of A of all genotypes at re-elevated temperatures, while maintaining ϕ_{PSII} values comparable to those of *Mxg*. This suggests that the low F_v/F_m in the field are a result of heterogeneity in field conditions.

Leaf elongation negatively correlated with carbon assimilation under chilling conditions. *Msa* accessions RU2012-114, RU2012-112, RU2012-069, RU2012-073 which exhibited the lowest reductions of photosynthetic capacity over the 15-day chilling treatment also experiencing the highest levels of reduction in leaf elongation under these conditions. On the other hand, *Mxg* experienced the lowest reduction in leaf elongation under chilling conditions, although it still experienced a sizeable 88% reduction. Reductions in leaf elongation of *Mxg* at 10°/5° C corresponded with those observed by Glowacka *et al*. (2014), while observed values in *Msa* differed from the findings of that study, likely due to the widely different origins of the *Msa* accession in each study from that of *Mxg*. Although the source-sink relationship in these accessions under severe chilling temperatures is not currently known, it is possible that in the

Siberian *Msa* accessions high photosynthetic rates correspond with starch/sugars transport to the rhizome, such as those recorded by Purdy *et al*. (2013), without spending resources on vegetative growth. Environmental factors and plant growth could have a big effect on leaf elongation, in particular, the amount of starch reserves in the rhizomes could play a big role in leaf elongation when low temps reduce A.

In the recovery of photosynthetic rates under mild chilling conditions, Wang *et al*. (2008) showed that doubling PPDK contents and maintenance of rubisco contents over a 14-day period of chilling at 14° C allows *Mxg* to recover nearly 100% photosynthetic rates. In contrast, in this study under severe chilling conditions *Mxg* did not regain photosynthetic capacity over 15 days at 10°/5° C. Here the rates of decrease in A of both *Msa* accessions and *Mxg* were reduced but did not recover as the chilling treatment progressed over time, consistent with the observations of Friesen *et al*. (2014) under severe chilling conditions. In our study, the Siberian *Msa* accessions RU2012-114 and RU2012-112 only showed small intermittent increases in A, while *Mxg* did not show any recovery under chilling temperatures. Friesen *et al*. (2014) recorded similar drops in A with *Mxg* photosynthetic rates decreasing by about 62% after six days of severe chilling at 12°/5° C without showing any sign of recovery under chilling temperatures in comparison to those observed at 25° C. These findings correspond with the values gained in this study, where after 15 days of chilling at 10° C *Mxg*'s photosynthetic rate decreased by 83% without recovery. Additionally, Glowacka *et al*. (2014) and Glowacka et al. (2015b) showed that during the 11 days of severe chilling at 10°/5° C *Mxg*'s photosynthetic rates only rebound slightly under chilling temperatures in comparison to those observed at 25° C, with photosynthetic rates dropping by as much as 60% and 70%, respectively by the 11th day of chilling.

At present the mechanism for tolerance of severe chilling temperatures in *Miscanthus* remains unexplored. Is it possible that the mechanism for photosynthetic tolerance of severe chilling temperatures in the Siberian *Msa* accessions can be similar to the one suggested in *Mxg* under mild chilling temperatures? Can regulation of PPDK and rubisco contents (Du *et al*., 1999b; Naidu, *et al*., 2004; Wang *et al*., 2008), thylakoid protein regulation (Fryer et al., 1995; Spence *et al*. 2014), substantial accumulation of zeaxanthin (Farage *et al*. 2006), and expression of reactive oxygen-scavenging enzymes (Jahnke *et al*., 1991; Fryer *at al*., 1998; Ort and Baker, 2002) account for mitigation of damage under severe chilling temperatures in the Siberian *Msa* accessions?

Following the overnight freezing treatment all 7 Siberian *Msa* accessions did not differ significantly from *Mxg* both in terms of retention of A and ϕ_{PSII}. As discussed by Friesen *et al*.

(2014), frost tolerance is not related to chilling tolerance in plants. Frost tolerance is associated with mitigating dehydration, damage to proteins, maintenance of membranes, and scavenging reactive oxygen species (Ruelland *et al*., 2009). Although the Siberia-collected *Msa* did not out-perform *Mxg* in terms of retention of photosynthetic rates, none of the accessions suffered visible damage or death as reported in other studies involving frost damage to *Msa* collected from lower latitudes (Zub and Brancourt-Hulmel, 2010; Friesen *et al*., 2014; Glowacka *et al*. 2015a).

In conclusion, the phenotypic evaluation of the Siberian *Msa* germplasm in this study proved to be a fruitful endeavor, supporting the hypotheses that individuals with superior C4 tolerance of severe chilling temperatures and frost can be found in the Siberian *Miscanthus* germplasm collected in high northern latitudes. Of note was *Msa* accession RU2012-114, which matched and out-performed *Mxg* across the board. Not only did it retain photosynthetic rates higher than *Mxg* under severe chilling conditions, it also maintained higher photosynthetic rates under warm temperatures before exposure to the severe chilling temperatures. Maintenance of high photosynthetic rates at warm temperatures is a desirable trait regardless of chilling tolerance and this addresses a key challenge for breeders to retain desirable traits in domesticated crops while introducing desirable traits from wild germplasm. This accession may prove to be a worthy parental line for the introgression of advantageous novel loci and alleles involved in C4 chilling tolerance into novel *Mxg* hybrids and 'miscanes'. Additionally, if crossed with a southern-adapted chilling-intolerant *Msa*, RU2012-114 can be very useful in the establishment of biparental mapping populations for future association studies.

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TABLES & FIGURES

Table 1 List and collection sites of 91 Siberian *Miscanthus sacchariflorus* accessions for which F_v/F_m measurements were taken between June 28 and July 1 at the Foulum Research Center, Denmark.

Accession Identifier	Collection site latitude	Collection site longitude	Collection site elevation
RU2012-019	48.52554° N	134.93182° E	34 m
RU2012-020	48.52549° N	134.93112° E	36 m
RU2012-025	48.52153° N	134.95441° E	33 m
RU2012-027	48.5213° N	134.95441° E	35 m
RU2012-028	48.52137° N	134.95447° E	34 m
RU2012-029	48.52453° N	134.95181° E	37 m
RU2012-034	49.06441° N	136.51799° E	23 m
RU2012-035	49.06432° N	136.51791° E	24 m
RU2012-037	49.06464° N	136.51773° E	23 m
RU2012-045	49.33253° N	136.52118° E	21 m
RU2012-046	49.33235° N	136.52069° E	25 m
RU2012-047	49.32945° N	136.51761° E	24 m
RU2012-048	49.3296° N	136.517141° E	28 m
RU2012-049	49.32977° N	136.51747° E	29 m
RU2012-050	48.9179° N	136.22987° E	26 m
RU2012-059	48.59626° N	135.59987° E	116 m
RU2012-060	48.59621° N	135.59979° E	116 m
RU2012-061	48.59811° N	135.59952° E	116 m
RU2012-062	48.59607° N	135.59433° E	117 m
RU2012-064	48.46698° N	135.44012° E	30 m
RU2012-068	48.46773° N	135.43912° E	32 m
RU2012-069	48.62491° N	135.13335° E	34 m
RU2012-070	48.62396° N	135.13426° E	29 m
RU2012-073	48.62415° N	135.13425° E	28 m
RU2012-074	48.62651° N	135.1432° E	32 m
RU2012-078	48.66663° N	132.98172° E	69 m
RU2012-079	48.66692° N	132.98165° E	69 m
RU2012-080	48.66696° N	132.98195° E	68 m
RU2012-081	48.66699° N	132.98215° E	69 m
RU2012-082	48.58871° N	133.02688° E	60 m
RU2012-083	48.44911° N	133.07716° E	80 m
RU2012-084	48.36573° N	133.13956° E	47 m
RU2012-091	48.29384° N	133.17572° E	35 m
RU2012-092	48.29395° N	133.17581° E	38 m
RU2012-093	48.294° N	133.1758° E	41 m
RU2012-094	48.29412° N	133.17593° E	44 m

Table 1 continued

Accession Identifier	Collection site latitude	Collection site longitude	Collection site elevation
RU2012-095	48.2915° N	133.17241° E	45 m
RU2012-099	48.70922° N	133.19159° E	67 m
RU2012-100	48.70947° N	133.1916° E	66 m
RU2012-101	48.70993° N	133.19168° E	66 m
RU2012-102	48.70965° N	133.19217° E	66 m
RU2012-104	48.59444° N	136.63362° E	49 m
RU2012-105	48.59678° N	133.63899° E	52 m
RU2012-110	48.58725° N	133.93983° E	46 m
RU2012-111	48.58809° N	133.94029° E	47 m
RU2012-112	48.58787° N	133.93944° E	45 m
RU2012-114	48.6093° N	134.21509° E	42 m
RU2012-116	48.57198° N	134.42264° E	37 m
RU2012-120	48.57172° N	134.423° E	38 m
RU2012-121	48.54032° N	134.71992° E	34 m
RU2012-124	48.36315° N	135.07053° E	67 m
RU2012-125	48.36325° N	135.0705° E	72 m
RU2012-132	47.94595° N	135.07269° E	58 m
RU2012-133	47.94543° N	135.07243° E	58 m
RU2012-135	47.89243° N	135.00781° E	49 m
RU2012-136	47.89221° N	135.00757° E	51 m
RU2012-137	47.89191° N	135.00688° E	47 m
RU2012-138	47.5072° N	134.74229° E	60 m
RU2012-144	47.21595° N	134.36623° E	90 m
RU2012-145	47.21572° N	134.36598° E	87 m
RU2012-147	47.21481° N	134.36682° E	83 m
RU2012-151	46.86573° N	134.26971° E	87 m
RU2012-154	46.42865° N	134.25366° E	72 m
RU2012-156	46.42876° N	134.28346° E	73 m
RU2012-157	46.42882° N	134.25372° E	74 m
RU2012-158	46.42939° N	134.25443° E	74 m
RU2012-159	46.42645° N	134.25317° E	74 m
RU2012-161	46.11338° N	133.93321° E	79 m
RU2012-167	45.34602° N	133.55511° E	89 m
RU2012-168	45.34602° N	133.55545° E	88 m
RU2012-169	45.34596° N	133.55589° E	89 m
RU2012-171	45.18425° N	133.45932° E	118 m
RU2012-177	43.97194° N	132.1884° E	110 m
RU2012-178	43.97172° N	132.1884° E	109 m
RU2012-179	43.97126° N	132.18762° E	113 m

Table 1 continued

Accession Identifier	Collection site latitude	Collection site longitude	Collection site elevation
RU2012-182	43.75277° N	132.08032° E	59 m
RU2012-183	43.75422° N	132.0818° E	74 m
RU2012-189	43.93143° N	131.88283° E	47 m
RU2012-190	44.17197° N	131.70364° E	136 m
RU2012-193	44.43401° N	131.40192° E	174 m
RU2012-194	44.43468° N	131.40121° E	175 m
RU2012-195	44.43479° N	131.40082° E	176 m
RU2012-200	44.89875° N	131.5905° E	137 m
RU2012-201	44.89843° N	131.58983° E	139 m
RU2012-202	44.89891° N	131.59099° E	139 m
RU2012-205	45.19675° N	131.97615° E	77 m
RU2012-206	45.19693° N	131.97554° E	75 m
RU2012-211	44.69672° N	131.97116° E	80 m
RU2012-212	44.69731° N	131.97177° E	82 m
RU2012-213	44.69729° N	131.97246° E	82 m
RU2012-214	44.69109° N	131.95929° E	113 m
RU2012-215	44.69988° N	131.97716° E	88 m

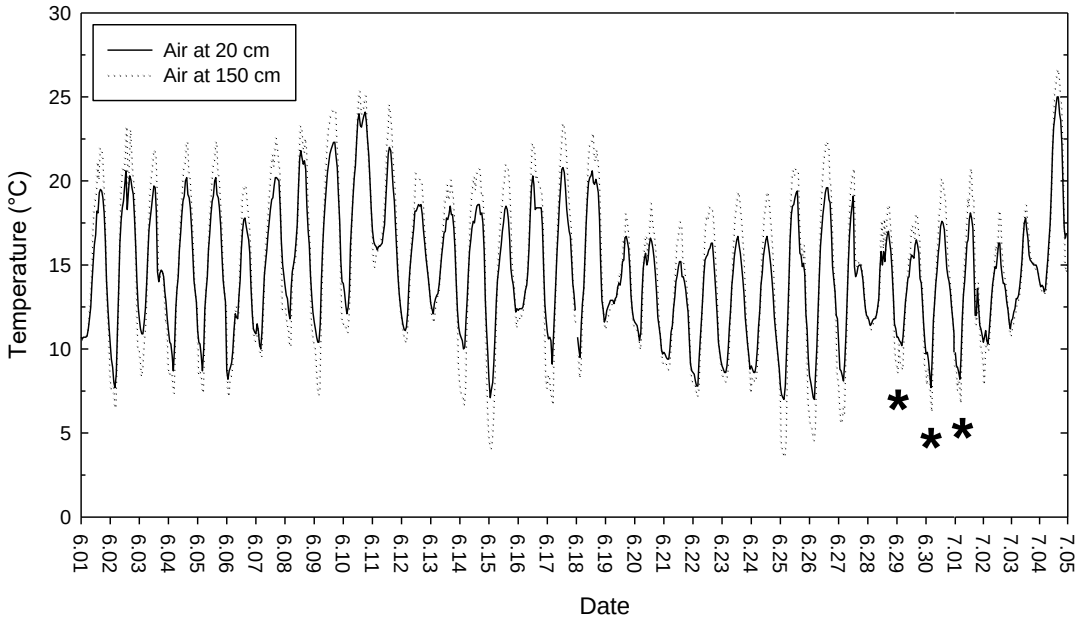

Fig. 1 Air temperatures during the months of June and July 2014. Temperatures were measured at 10 minute intervals and averaged per hour at 20 cm (solid line) and 150 cm (dotted line) above the surface of the soil in the Foulum Research center. Asterisks indicate nights during which F_v/F_m measurements were made in the field. On June 28th 39 *Msa* accessions were measured along with *Mxg*, on June 30th 42 *Msa* accessions were measured along with *Mxg*, and on July 1st 11 new accessions along with *Mxg* were measured along with 20 of the top performing accessions from June 28th and June 30th. *Msa= Miscanthus sacchariflorus, Mxg= Miscanthus x giganteus*.

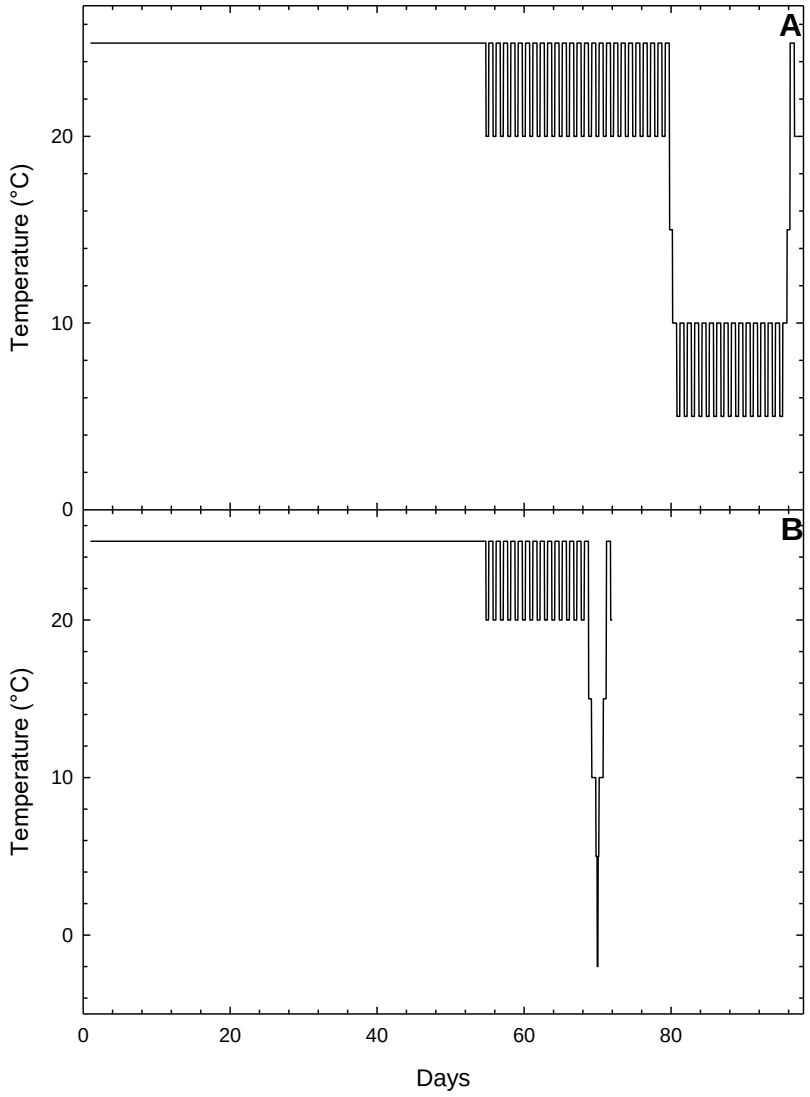

Fig. 2 Graphical representation of the temperature scheme at which the potted miscanthus was grown and measured during the (A) 15-day chilling treatment and (B) overnight frost treatment. Each treatment was measured on a separate set of plants - for both treatments the same seven Siberian *Msa* accessions were chosen according to their F_v/F_m measurements during a chilling period in Foulum, Denmark during the summer of 2014. Plants were propagated from rhizome and grown in the greenhouse for 53 days before transfer to growth cabinets. Four potted plants for each *Msa* accession along with 4 pots of *Mxg* were acclimated (separately for each treatment) to growth cabinet conditions for 23 days for the 15-day chilling treatment and 10 days for the overnight frost treatment at 25° C light/20° C dark conditions, after which a period of 4 days of sequential control measurements (gas exchange, fluorescence, and leaf elongation) were carried at 25° C to ensure stability of carbon assimilation capacities. The (A) 15-day chilling treatment involved a 15-day chilling period (10° C light/ 5° C dark) during which gas exchange, fluorescence, and leaf elongation rates were measured every 2 days at 10° C, followed by 1 day of measurements at re-elevated temperatures (25° C). The (B) overnight frost treatment involved a two-day chilling period (10° C light/ 5° C dark) during which the plants were exposed to -2° C for three consecutive hours during the dark period. Gas exchange and fluorescence were measured every day at 10° C, followed by 1 day of these measurements at re-elevated temperatures (25° C). *Msa*= Miscanthus sacchariflorus, *Mxg*= Miscanthus x giganteus.

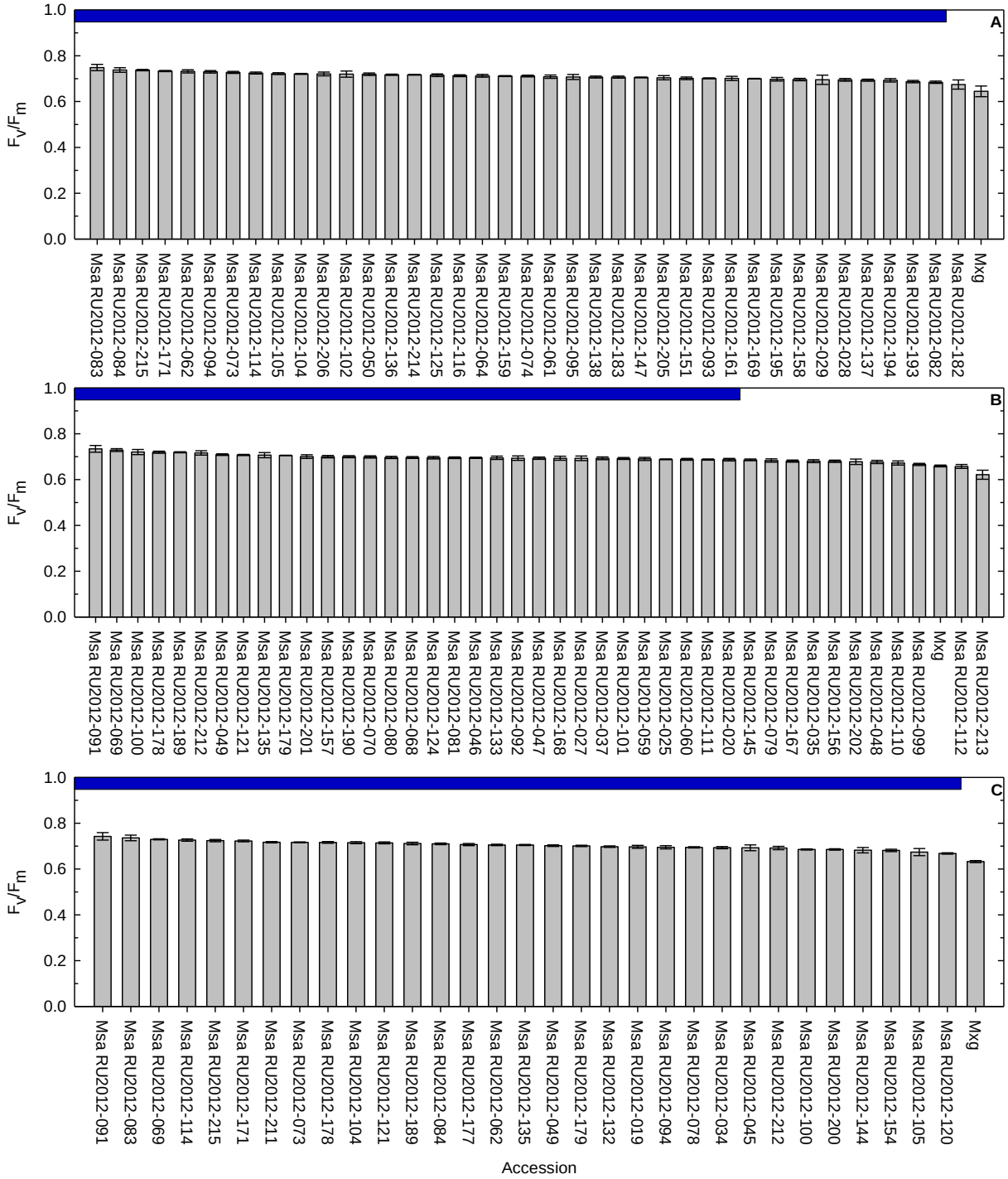

Fig. 3 Maximum dark-adapted quantum efficiency of photosystem II (Fv/Fm) of Siberian *Miscanthus sacchariflorus* accessions and *Miscanthus x giganteus* measured in the field overnight on (A) June 29th (B) June 30th, and (C) July 1st in Foulum, Denmark. Means (n=4) and standard error bars are shown. Blue bars represent upper li0mit mean comparisons to *Mxg* at each day by post-hoc Dunnett test (p <0.1). On June 28th 39 *Msa* accessions were measured along with *Mxg*, on June 30th 42 *Msa* accessions were measured along with *Mxg*, and on July 1st 11 new accessions along with *Mxg* were measured along with 20 of the top performing accessions from June 28th and June 30th. *Msa= Miscanthus sacchariflorus, Mxg= Miscanthus x giganteus*.

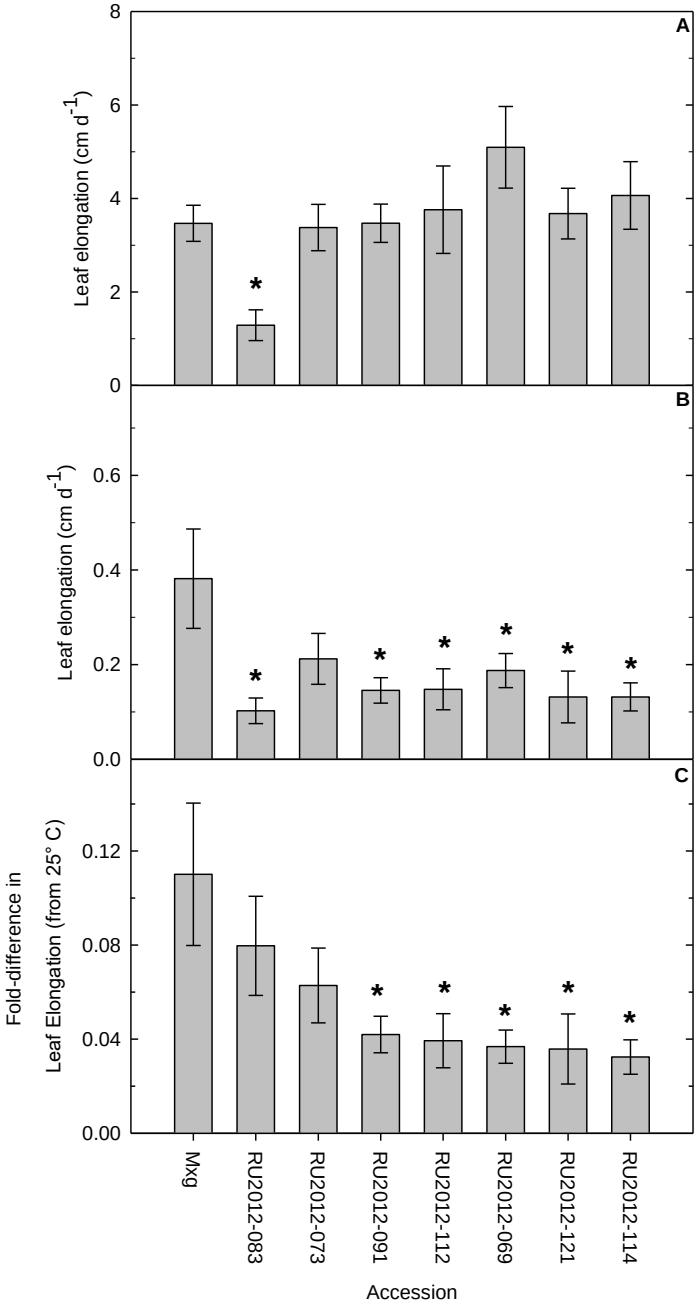

Fig. 4 The effect of a 15-day chilling treatment on the rate of leaf elongation at (A) warm conditions at 25° C (B) chilling temperatures at 10° C , and (C) The fold-difference between rates of elongation at chilling temperature and warm temperature conditions for Siberian *Msa* accessions and *Mxg*. Means (n=4) and standard error bars are shown, accessions are arranged in all panels according to the fold-difference between rates of elongation at chilling temperature and warm temperature conditions (panel C) from highest to lowest. Leaf elongation rates were measured every day at 25° C light-time/ 20° C dark-time temperature over a five day period before the chilling treatment and every two days during the 10° C light-time/ 5° C dark-time temperature 15-day chilling treatment. Asterisks above bars indicate significant mean differences from *Mxg* at each growing temperature by post-hoc Dunnett test ($p < 0.1$). Plants were grown at 25° /20° C day/night and 10° /5° C day/night , and a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity. *Mxg= Miscanthus x giganteus, Msa= Miscanthus sacchariflorus*.

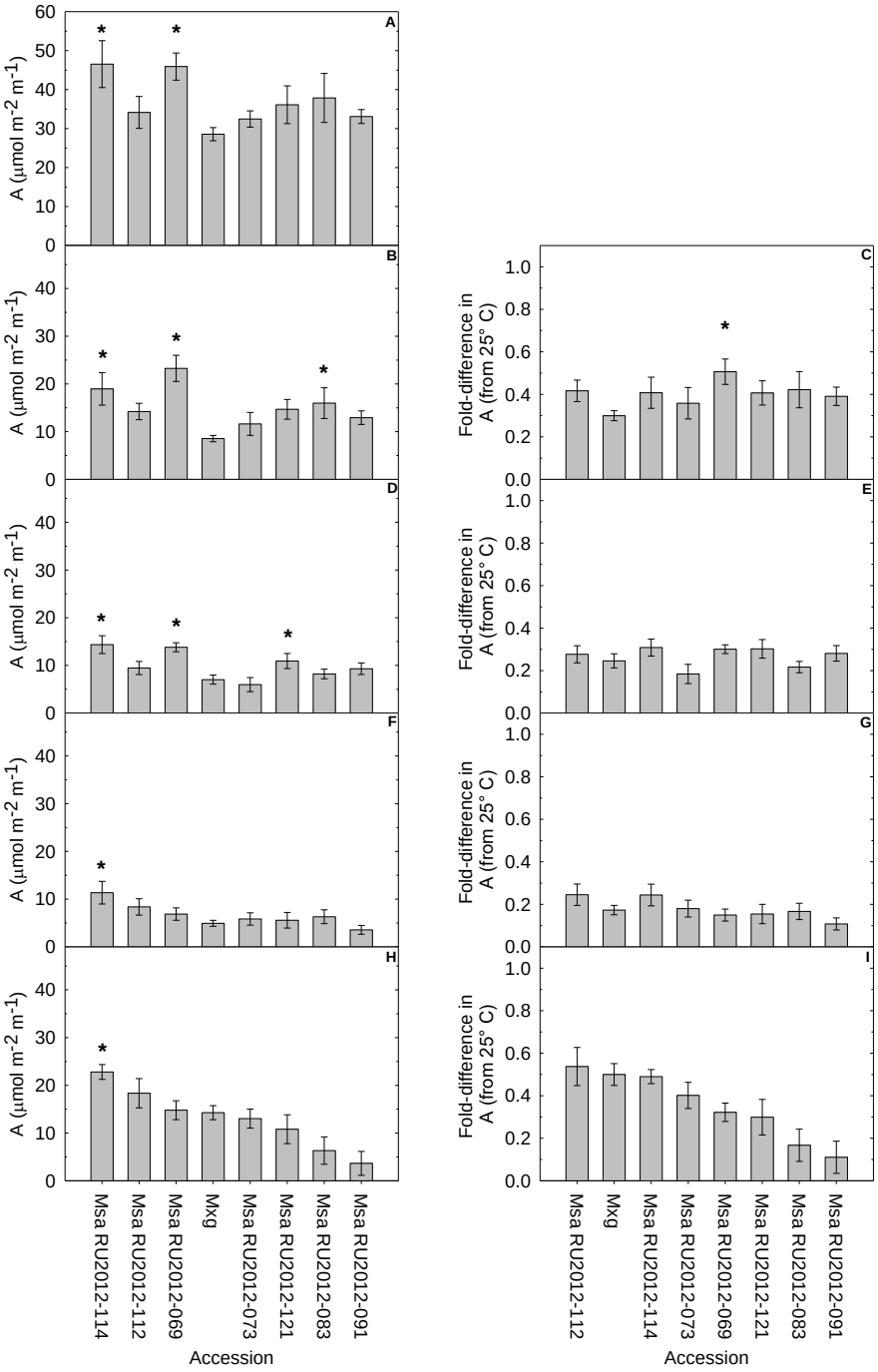

Fig. 5 The effect of a 15-day chilling treatment on leaf carbon assimilation rates under saturating light (2000 µmol m^{-2} s^{-1}) for each Siberian *Msa* accession and *Mxg*. The left column shows means (n=3 to 4) and standard error of carbon assimilation rates, and the right graph column shows means (n=3 to 4) and standard error of the rate of carbon assimilation relative to the measurements at 25° C before initiation of the chilling treatment. Carbon assimilation rates (A) at 25° C before initiation of chilling treatment, (B-C) at 10° C during the first day of chilling treatment, (D-E) at 10° C 8 days after initiation of chilling treatment, (F-G) at 10° C 15 days after initiation of chilling treatment, and (H-I) at 25° C on the first day after re-elevation of temperatures. Asterisks above bars indicate significantly higher means from *Mxg* at each day by post-hoc Dunnett test ($p \leq 0.1$). In all panels per each graph column, accessions are ordered by values obtained during the last day of measurements at re-elevated temperatures at 25° C (panels H and I, respectively). Plants were grown at 25° /20° C day/night and 10° /5° C day/night , and a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity. *Mxg= Miscanthus x giganteus, Msa= Miscanthus sacchariflorus*.

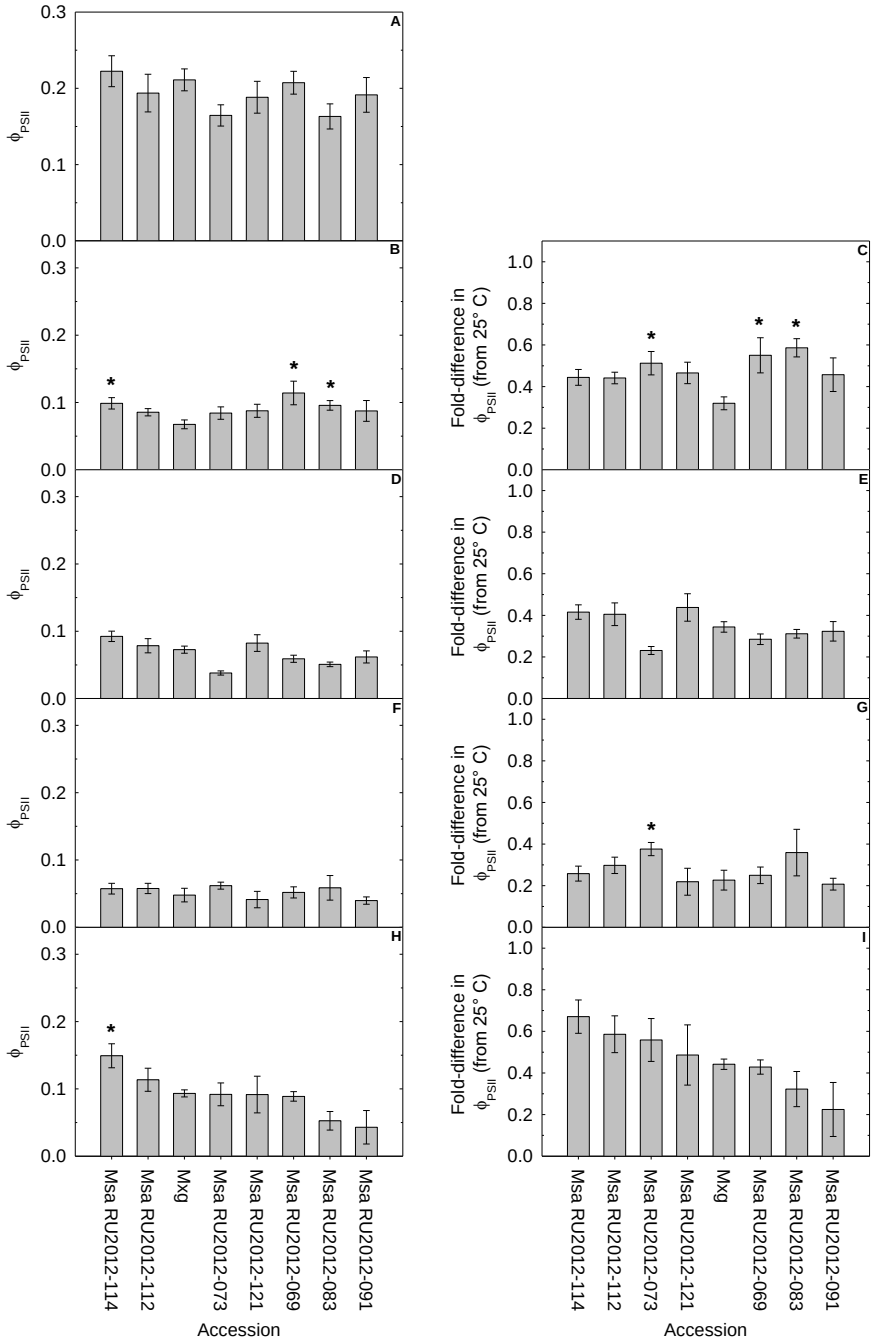

Fig. 6 The effect of a 15-day chilling treatment on quantum yield of photosystem II (ϕ_{PSII}) measured at 2000 µmol photons m^{-2} s^{-1} for each Siberian *Msa* accession and *Mxg*. The left column depicts means (n=3 to 4) and standard error of ϕ_{PSII}, and the right graph column depicts means (n=3 to 4) and standard error of ϕ_{PSII} relative to the measurements at 25° C before initiation of the chilling treatment. ϕ_{PSII} values (A) at 25° C before initiation of chilling treatment, (B-C) at 10° C during the first day of chilling treatment, (D-E) at 10° C 8 days after initiation of chilling treatment, (F-G) at 10° C 15 days after initiation of chilling treatment, and (H-I) at 25° C on the first day after re-elevation of temperatures. Asterisks above bars indicate significantly higher means from *Mxg* at each day by post-hoc Dunnett test ($p \leq 0.1$). In all panels per each graph column, accessions are ordered by values obtained during the last day of measurements at re-elevated temperatures at 25° C (panels H and I, respectively). Plants were grown at 25° /20° C day/night and 10° /5° C day/night , and a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity. *Mxg= Miscanthus x giganteus, Msa= Miscanthus sacchariflorus*.

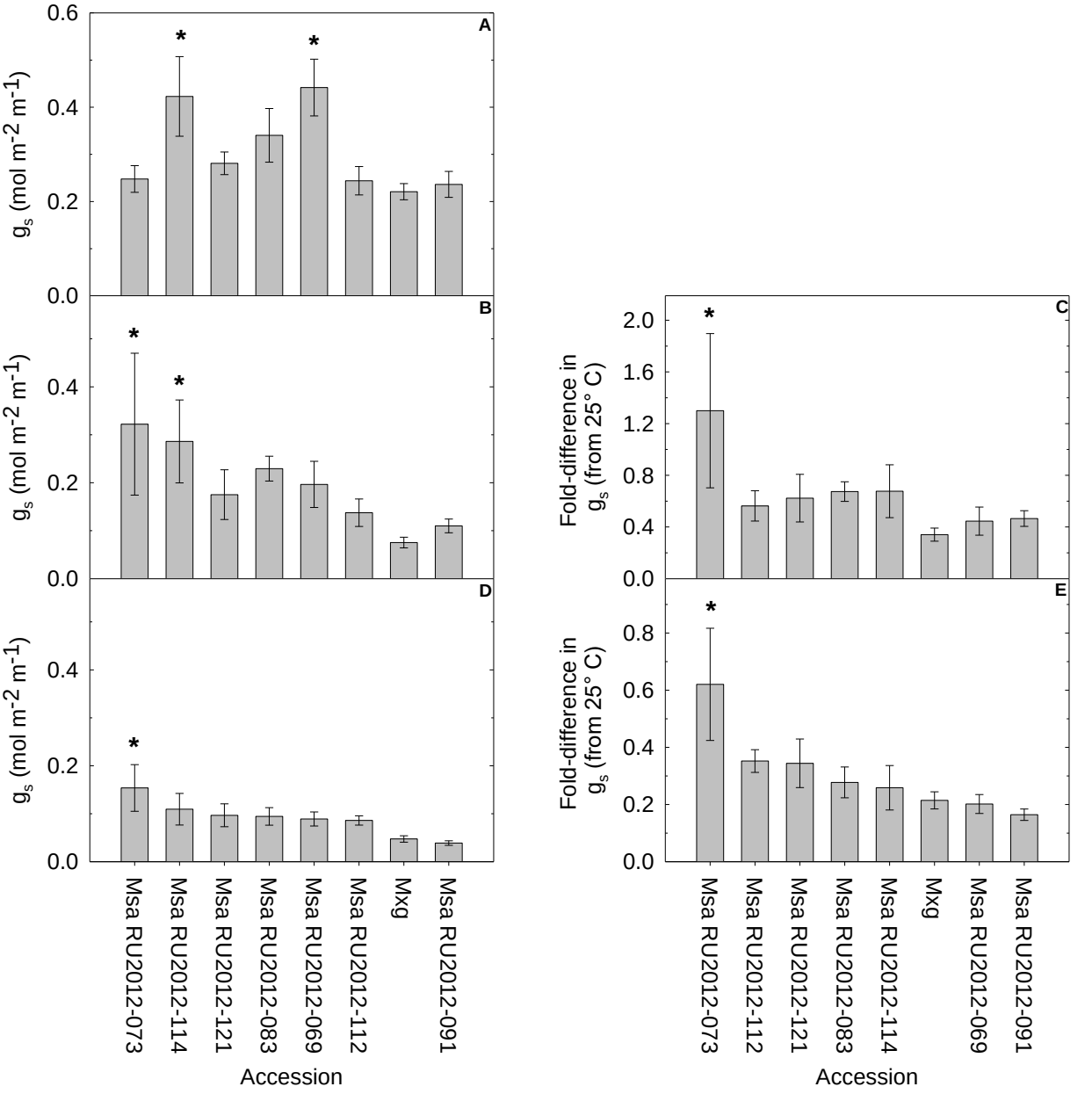

Fig. 7 The effect of a 15-day chilling treatment on stomatal conductance of water vapor (g_s) measured at 2000 µmol photons m^{-2} s^{-1} for each Siberian *Msa* accession and *Mxg*. The left column depicts means (n=3 to 4) and standard error of g_s, and the right graph column depicts means (n=3 to 4) and standard error of g_s relative to the measurements at 25° C before initiation of the chilling treatment. Stomatal conductance values (A) at 25° C before initiation of chilling treatment, (B-C) at 10° C during the first day of chilling treatment, and (D-E) at 10° C 15 days after initiation of chilling treatment. Asterisks above bars indicate significant mean differences from *Mxg* at each day by post-hoc Dunnett test ($p < 0.1$). In all panels per each graph column, accessions are ordered by values obtained during the last day of measurements at 10° C (panels D and E, respectively). Plants were grown at 25° /20° C day/night and 10° /5° C day/night , and a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity. *Mxg= Miscanthus x giganteus, Msa= Miscanthus sacchariflorus*.

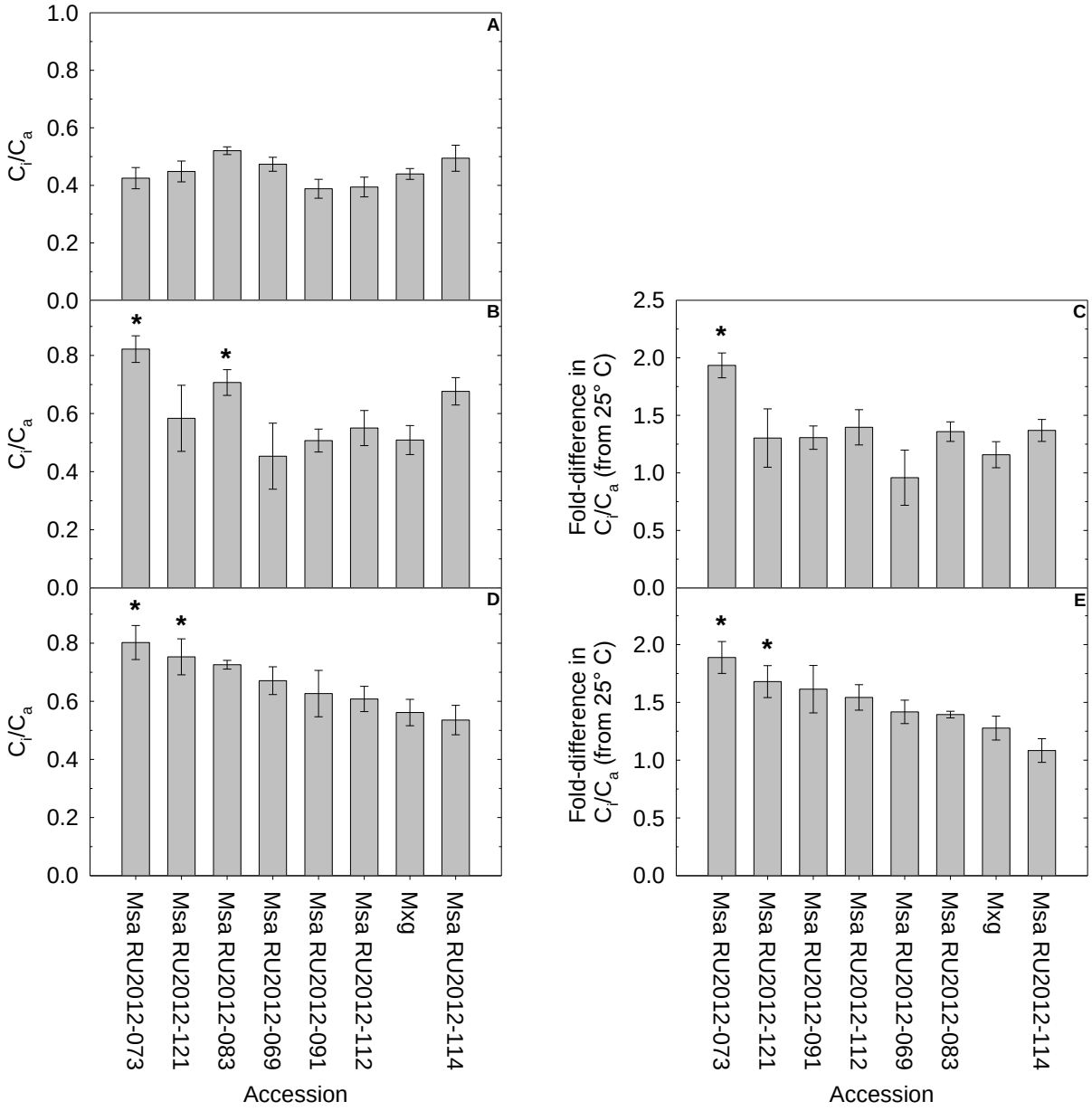

Fig. 8 The effect of a 15-day chilling treatment on the ratio of intercellular to atmospheric CO_2 (C_i/C_a) measured at 2000 µmol photons m^{-2} s^{-1} for each Siberian *Msa* accession and *Mxg*. The left column depicts means (n=3 to 4) and standard error of C_i/C_a, and the right graph column depicts means (n=3 to 4) and standard error of C_i/C_a relative to the measurements at 25° C before initiation of the chilling treatment. Ratio of intercellular to atmospheric CO_2 (A) at 25° C before initiation of chilling treatment, (B-C) at 10° C during the first day of chilling treatment, and (D-E) at 10° C 15 days after initiation of chilling treatment. Asterisks above bars indicate significant mean differences from *Mxg* at each day by post-hoc Dunnett test ($p< 0.1$). In all panels per each graph column, accessions are ordered by values obtained during the last day of measurements at 10° C (panels D and E, respectively). Plants were grown at 25° /20° C day/night and 10° /5° C day/night , and a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity. *Mxg= Miscanthus x giganteus, Msa= Miscanthus sacchariflorus*.

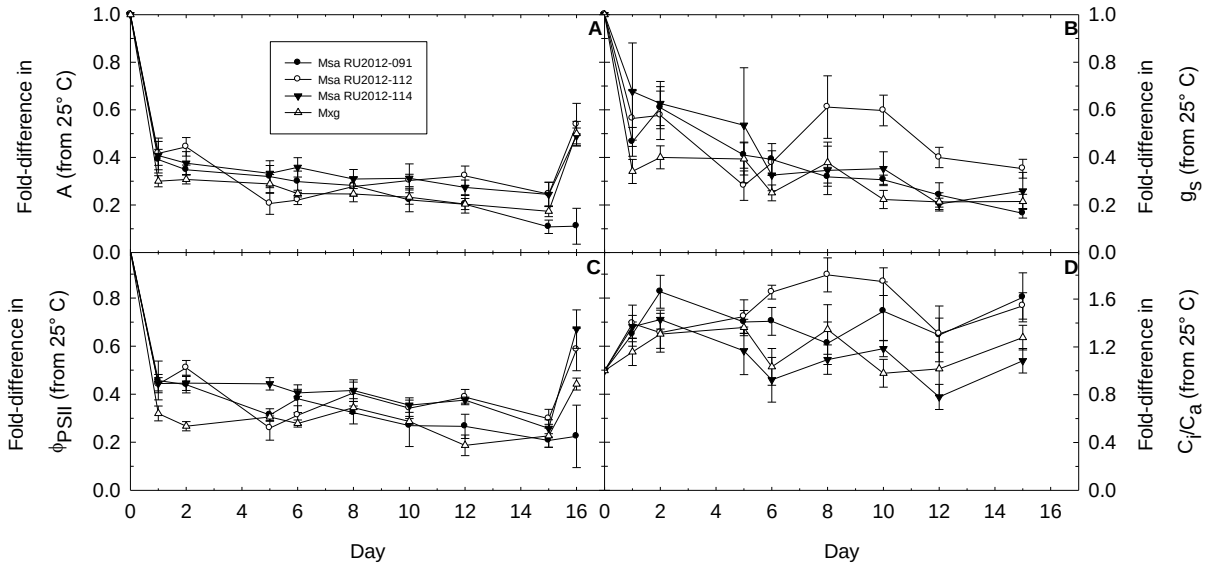

Fig. 9 Fold-differences from control measurements at 25° C in (A) carbon assimilation rates (A) , (B) stomatal conductance to water vapor (g_s), (C) Quantum yield of photosystem II (ϕ_{PSII}), and Ratio of intercellular to atmospheric CO_2 (C_i/C_a) as a function exposure of plants to chilling temperatures over a 15-day chilling treatment (10 ° /5 °C day/night). Means (n=3 to 4) ± Standard error bars are shown for the two top-performing Siberian *Msa* accessions, worst-performing Siberian *Msa* accession, and *Mxg*. Plants were grown at 25 ° /20 °C day/night under controlled conditions for 27 days (day 0 is the control measurements at warm conditions) and then exposed to a 15-day 10 ° /5 °C day/night chilling treatment (days 1-15), followed by one day of re-elevated temperatures to 25 °C (day 16). Environmental factors were controlled at 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1}, and 75% relative air humidity. Measurements were taken during the daytime. *Mxg= Miscanthus x giganteus, Msa= Miscanthus sacchariflorus*.

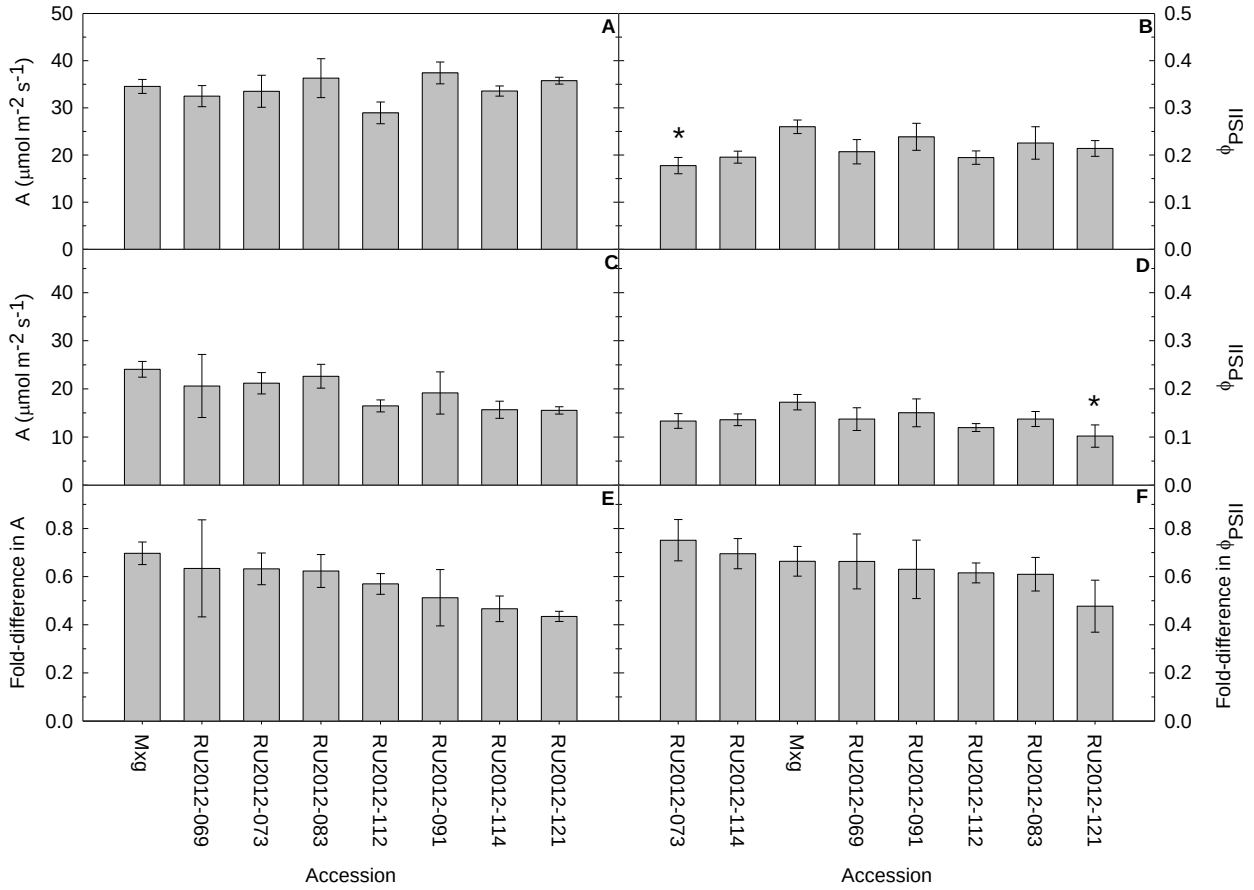

Fig. 10 The effect of an overnight freezing treatment on leaf carbon assimilation rates (A) in the left columns and quantum yield of photosystem II (ϕ_{PSII}) in the right columns under saturating light (2000 µmol m^{-2} s^{-1}) for each Siberian *Msa* accession and *Mxg*. Leaf carbon assimilation rates in (A) warm conditions prior to the freezing treatment, (C) in warm conditions after the chilling treatment, and (E) the fold-difference in A after the freezing treatment. Quantum yield of photosystem II in (B) warm conditions prior to the freezing treatment, (D) in warm conditions after the chilling treatment, and (F) the fold-difference in ϕ_{PSII} after the freezing treatment. Means (n=4) and standard error bars are shown, accessions are arranged in all panels according to the fold-difference between rates A and ϕ_{PSII} in warm conditions after the freezing treatment (panels E and F, respectively) from highest to lowest. Asterisks above bars indicate significant mean differences from *Mxg* at each growing temperature by post-hoc Dunnett test ($p< 0.1$). The plants were kept at warm conditions 25 °C light /20 °C for 14 days, followed by two days of chilling at 10° /5° C day/night , during the first night of chilling they were exposed to a three-hour freezing treatment at -2° C. Plants were grown at a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity. *Mxg= Miscanthus x giganteus, Msa= Miscanthus sacchariflorus*.

CHAPTER 3

EARLY-GENERATION TESTING FOR EXCEPTIONAL C4 PHOTOSYNTHETIC CHILLING TOLERANCE IN ENERGYCANE HYBRIDS

ABSTRACT

Energycane is a bioenergy feedstock candidate for biomass production in warm temperate and subtropical climates of the United States, but photosynthetic limitations of the C4 cycle at suboptimal temperatures limit the range at which it can be optimally cultivated. The development of chilling-tolerant varieties would permit extension of the growing season and mitigation of losses to biomass yields at higher latitudes. The objective of this study is an early-generation screening of *Saccharum* spp. and *Erianthus* spp. hybrids for superior photosynthetic chilling tolerance. In this experiment the photosynthetic chilling tolerance of 26 energycane hybrids derived from 10 different crosses was evaluated over the course of 15 days under controlled environment conditions. The chilling treatment involved the transfer of plants grown under warm temperatures 25°/20° C day/night to controlled conditions under 15°/10° C day/night temperatures for 15 days, followed by a one day of re-elevation of temperatures to 25° C. The majority of energycane genotypes investigated here showed higher or comparable chilling tolerance than the chilling-intolerant historically cultivated *S. officinarum* 'LA Purple', while three energycane F1 hybrids (HB07-3452-4, HB07-3073-6, and HB07-3329-5) maintained the highest rates of leaf carbon assimilation (A) and quantum yield of photosystem II (ϕ_{PSII}) over the chilling period and at the re-elevation of temperatures. In conclusion, three energycane genotypes were identified to have superior chilling tolerance to that of the chilling-intolerant *S. officinarum* 'LA Purple', and may produce favorable results in future crossing efforts for superior chilling-tolerance in energycane variety development.

INTRODUCTION

In recent years there has been an emphasis on meeting the U.S. federal government's mandate to increase the production of bioenergy in the United States over the upcoming decades. Sugarcane grown for its lignocellulosic biomass rather than sugar content, termed as 'energycane', has been suggested as a feedstock for the biofuel industry in tropical and subtropical parts of the United States (Bransby *et al*., 2010). Due to the high yield potential of energycane, there would be an obvious benefit to expanding the bounds of its cultivation in the continental U.S. from Louisiana and Florida to states such as Georgia, Alabama, Mississippi,

and S. Carolina. At present, sugarcane is the most productive bioenergy crop in terms of overall bio-ethanol yields, but due to chilling-intolerance of many varieties its cultivation is restricted to tropical and subtropical climates with a relatively narrow range of optimum temperature tolerance between 25-35° C (Clements, 1980; Du *et al*., 1999a-b). Due to sugarcane's long growing season, its chilling intolerance often makes it susceptible to reductions in yield when cultivated in higher latitudes or elevations (Grantz, 1989). Low temperatures put a heavy vegetative growth limitation on sugarcane (Verret and Das, 1927), and chilling events have been shown to cause chlorosis in leaves (Faris, 1926), although early studies have suggested that sprouting may occur at temperatures as low as 6° (Sartorius, 1929). The exposure of sugarcane to chilling temperatures is accompanied with reduced sucrose accumulation in stems (D'Hont *et al*. 2008) and failure to produce high shoot biomass (Ebrahim *et al*., 1998; Inman-Bamber *et al*., 2010; Campbell *et al*., 1998); and sugarcane leaf elongation has been shown to completely stop at 10° C, while tillering ceases at 16° C (Inman-Barber *et al*., 2014). The chilling-susceptibility of sugarcane is highly variety-specific, with some varieties experiencing more damage and reductions of photosynthetic capacities than others (Du, 1999a).

To date, there have been a low number of publications on the response of photosynthesis in sugarcane to chilling temperatures. In one study, carbon assimilation rates of 13 sugarcane varieties decreased by nearly 55-75% with the drop of leaf temperatures from 25° C to 15°C, and carbon assimilation rates extrapolate to zero around 10° C where sugarcane is susceptible to chilling injury (Grantz, 1989; Nose *et al.*, 1994; Sage *et al.*, 2014). Conversely, the chilling sensitivity of sugarcane varies seasonally, and seemingly increases in the spring and winter (Grantz, 1989), corresponding with the observation of increased gene expression as RNA of pyruvate orthophosphate dikinase (PPDK) and NADP-dependent malic enzyme (NADP-ME) in some sugarcane cultivars in response to chilling (Nogueira *et al*., 2003). In the analysis of PPDK enzyme activity of three sugarcane varieties, the activity of extracted PPDK proteins decreased by nearly 50% corresponding with temperature decreases from 25° to 15° C, suggesting a chilling-lability of the enzyme (Du *et al*, 1999b), while photosynthetic chilling tolerance appears to be cultivar-dependent (Du *et al*., 1999a). Additionally, in a Brazilian sugarcane variety the net CO_2 assimilation rate (A) response to intercellular CO_2 concentration (Ci) shows that there is a significant decrease in the portion of the response curve that is limited by the maximum enzymatic velocity of PPDK and ribulose-1,5-bisphosphate carboxylase/ oxygenase (rubisco)(Friesen *et al*., 2014; Sage *et al*., 2014). This corresponds with observations in maize, another chilling intolerant C4 species, whose photosynthetic rates crash along with reductions rubisco and PPDK contents following exposure to chilling conditions

(Naidu *et al*. 2003; Naidu and Long, 2004; Wang *et al*., 2008). Accordingly, it has been observed in maize and *Miscanthus* that chilling intolerance results from inability to maintain sufficient total PPDK and rubisco contents, express/repair/degrade the chilling-susceptible D1 protein of photosystem II (PSII), and avoid photoinhibition through non-photochemical quenching (NPQ) that results from accumulation of zeaxanthin and anthocyanins (Farage *et al*., 2006; Grennan and Ort, 2007; Wang *et al*. 2008; Long and Spence, 2013). In the chilling-tolerant C4 grass *Miscanthus x giganteus*, substantial upregulation of PPDK protein, maintenance of rubisco protein concentrations, retention of quantum yield of photosystem II (ϕ_{PSII}), and accumulations of zeaxanthin have been observed after exposure to chilling, and are believed to be major components of C4 photosynthetic chilling tolerance (Wang *et al*., 2008; Farage *et al*., 2006), while increases in the concentrations of enzymes involved in scavenging reactive oxygen species and the water-water cycle have been shown in chilling-intolerant maize as damage avoidance/ mitigation mechanisms (Jahnke *et al*., 1991; Fryer *et al*., 1998; Ort and Baker, 2002).

In the effort to generate new chilling-adapted cultivars of sugarcane, breeders have integrated wide crosses between chilling-tolerant *S. spontaneum* and commercial varieties of *S. officinarum* into their breeding programs (Khan *et al*., 2013; Daniel and Roach, 1987). Some *S. spontaneum* genotypes are known for their superior chilling/freezing tolerance within the *Saccharum* genus (Brandes, 1940). Additionally some *Erianthus arundinaceus* and *Erianthus-Saccharum* hybrids have been shown to have superior tolerance of various abiotic stresses (Bakshi *et al*., 2001), while 'miscanes' generated through inter-genus hybridizations between *M. sinensis Saccharum spp*. have been shown to match the photosynthetic chilling tolerance of *Miscanthus x giganteus* at 10° C (Glowacka et al, 2015). As such, new superior varieties of sugarcane have been shown to out-perform commercial sugarcane varieties and even compete with Napier grass yields in southeastern U.S., yielding over 30 Mg h^{-1} in southern Georgia, which is a step towards the goal of cultivation of high biomass feedstock production in warm temperate climates (Hale *et al*., 2013; Knoll *et al*, 2013). Introgression of genes conferring chilling tolerance from wild germplasm into sugarcane is needed due to the inherent chilling intolerance of *S. officinarum.* Inter-species crosses with wild *S. spontaneum* and inter-genus crosses with the closely-related genera *Erianthus* and *Miscanthus* may facilitate the introduction of novel loci and alleles into superior sugarcane cultivars.

In this study, the photosynthetic chilling-tolerance of energycane genotypes generated through the USDA-ARS basic breeding program in Houma, Louisiana were evaluated against that of the *S. officinarum* 'LA Purple' over a 15-day chilling (15° C day/ 10° C night) period. Each

genotype was cloned from shoot-cuttings, and the clones were transferred to controlled environment chambers to further investigate each genotype's photosynthetic capacity under chilling conditions and short-term recovery from chilling damage. We tested the hypothesis that the progeny of crosses between superior sugarcane cultivars and chilling-tolerant *S. spontaneum* or *E. arundinaceous* will exhibit greater chilling tolerance than *S. officinarum* 'LA Purple' in terms of maintenance of light-saturated photosynthesis (A_{sat}) and quantum yield of photosystem II (ϕ_{PSII}).

MATERIALS AND METHODS

Plant material

Overall, 26 genotypes derived from 10 different crosses between superior lines and wild germplasm were included in this study. The energycane genotypes were acquired from the USDA-ARS basic sugarcane breeding program in Houma, Louisiana. Parental lines were chosen on the basis of yield (sugar per acre, tons of cane per acre, theoretical recoverable sucrose, biomass accumulation, and tiller generation), general disease resistance, and chilling tolerance. Genotypes were derived from various stages of the backcross possess, with material representing *Erianthus arundinaceous* polycrosses, F1 hybrids between commercial sugarcane varieties and wild relatives, F1' hybrids derived from F1 x F1 crosses, BC1 generations, and BC2 generations (Table 2). All genotypes have varying degrees of *Saccharum spontaneum* and *Erianthus* ancestry. *S. officinarum* 'LA Purple' (hereafter referred to as 'LA Purple'), the historically-cultivated variety in Louisiana, was chosen as a negative control for chilling tolerance based on the results of Glowacka *et al*. (2014). Prior to the present controlled-environment study, all genotypes were subjected to a 2-year overwinter chilling-tolerance screening in southern Arkansas between 2008 and 2010, and only the genotypes with the highest shoot-density-per-plant were selected for further evaluation in the present study.

Shoot segments of each genotype were cloned and grown in round 6 L pots (Classic 600 pots; Nursery Supplies, Inc., Orange, CA, USA) containing a peat/bark/perlite- based growing medium (Metro-Mix 900; Sun Gro Horticulture, Agawam, MA, USA). After emergence of new shoots, a fertilizer was added according to the manufacturer's instructions (Jack's Professional General Purpose Water Soluble Fertilizer, 20-20-20; JR Peters, Inc., Allentown, PA, USA) and additional iron was added prior to transfer to growth cabinets (ferrous sulphate heptahydrate; QC Corporation, Girardeau, MO, USA). Plants were grown in a controlled-environment greenhouse at the University of Illinois, Champaign-Urbana at ~25°/ ~19° C

day/night temperatures. Soil moisture content was maintained to field capacity by a daily watering regimen.

15-day chilling treatment

Leaf gas exchange and chlorophyll fluorescence were evaluated for all 26 sugarcane genotypes along with LA Purple under controlled environmental conditions over five temporally-separate trials between January 2014 and June 2015. Potted plants (3-5 pots) of each genotype were transferred from the greenhouse to a controlled-environment chamber (PGC20 Growth Chamber; Conviron, Winnipeg, Manitoba, Canada) with a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1}, 25 °C daytime/20 °C nighttime temperature, and relative humidity of 75%. After 13-24 days of acclimation to the growth chamber, leaf carbon uptake rates were analyzed for three to four consecutive days to evaluate the health of each plant, plants which showed marked drops in carbon assimilation rates, showed necrosis, or chlorosis were removed from the study. We then included 1 day of control measurements under warm conditions (25 ° /20 °C day/night), after which the temperature in the growth chamber was lowered to chilling temperatures (15°/10 °C day/night) for 15 days, and then returned to warm temperatures for 1 day (Fig. 2A). This 15-day period was chosen to emulate the type of chilling that developing plants might experience during vegetative growth in spring, and follows the bioassay developed by Wang *et al*. (2008) and used by Glowacka *et al*. (2014) and Glowacka *et al*. (2015). Aside from temperature, all other environmental conditions were unchanged. To avoid the confounding effects of heterogeneous environmental conditions within the growth cabinet, each plant within the growth cabinet was rotated every two days according to a fully randomized design.

Leaf photosynthetic gas exchange was measured in situ on the most recent fully expanded leaf, as indicated by ligule emergence, with an open gas exchange system incorporating CO_2 and water vapor analyzers (LI-6400; LI-COR, Lincoln, NE, USA). Leaf photosynthetic gas exchange was measured on the 3-4 days prior to the induction of the chilling treatment, and then measured on days 1, 5, and 15 of the chilling treatment. All measurements were taken between 3-8 h into the day portion of the photoperiod on light-adapted leaves by measuring precisely the same portion of the leaf on each measurement day. The leaf was enclosed in a controlled-environment cuvette with tracked temperature, humidity, and light. Measurements were conducted under ambient air (21% O_2) at 400 ppm CO_2 concentration, 1500 µmol m^{-2} s^{-1} photon flux, 75% relative humidity, and leaf temperature was controlled at the growth temperature for each treatment. Light-emitting diodes were used for actinic light (90%

red light, 630 nm; 10% blue light, 470 nm). The net CO_2 uptake rate per leaf area (A), stomatal conductance to water vapor (g_s), and intercellular CO_2 concentration (C_i) were calculated, as described in Bernacchi *et al*. (2003). Chlorophyll pulse amplitude fluorescence was measured simultaneously with leaf photosynthetic gas exchange by a fluorometer positioned in the cuvette lid (LI-6400-40; LI-COR, Lincoln, NE, USA). A multiphase flash protocol was used to maximize fluorescence emissions. The quantum yield of photosystem II under saturating light (Φ_{PSII}) was calculated as described by Maxwell and Johnson (2000). The cuvette was attached to the leaf for 10-20 minutes prior to measurement in order to achieve steady-state photosynthesis and stomatal response.

Data analysis

Statistical analyses were performed with SAS v. 9.3 (SAS Institute, Cary, NC, USA). All statistical analyses were performed at alpha 0.1. In order to maximize the sensitivity of statistical tests, the coefficients of variance of leaf carbon assimilation rates were calculated for all accessions across all measurement days in PROC MEANS and plotted in PROC UNIVARIATE. The six accessions whose coefficients of variance ranked in the top 25% of the distribution of coefficients of variance were removed from all statistical analyses in this experiment. The fixed main effect of accession on Fv/Fm was determined by a one-way analysis of variance (ANOVA), post-hoc Dunnett's mean separation tests were used to determine significant differences from 'LA Purple' per each measurement day. The fixed main effect of accession and time in the chilling/freezing treatment on all measures (leaf elongation rate, A, g_s, C_i/C_a, Φ_{PSII}, and relative changes of said variables) were determined by a two-way repeated-measures ANOVA in PROC MIXED with an unstructured covariance structure. In the presence of a significant main effect of accession on any variable, post-hoc Dunnett's mean separation tests were used to determine significant differences from 'LA Purple' per each measurement day.

RESULTS

Over the 15-day chilling treatment, sugarcane genotype had a significant main effect on all sugarcane genotypes for all gas exchange measurements (A, g_s, C_i/C_a) and ϕ_{PSII} ($p<0.1$), while days of chilling had significant effects on all 4 aforementioned variables ($p<0.0001$). Day-by-genotype interaction was not significant for any of these variables. Overall, three energycane accessions maintained higher rates of net leaf carbon assimilation (A) than 'LA Purple' after 15 days of chilling (15°/10° C day/night) and six energycane accessions maintained higher A than 'LA Purple' at return to warm temperatures (25°/20° C day/night) (Fig. 11F&H). Of note,

energycane accession HB07-3329-5 maintained higher A and lower reductions in A than 'LA Purple' throughout the 15-day chilling treatment and upon return warm conditions (Fig. 11), and accessions HB07-3452-4 and HB07-3073-6 maintained higher rates of A after 15 days of chilling and at return of temperatures to 25° C. Accessions HB07-3329-5, HB07-3452-4, and HB07-3073-6 maintained rates of A of 26.1 μmol m^{-2} s^{-1}, 25.5 μmol m^{-2} s^{-1}, and 25.6 μmol m^{-2} s^{-1} , respectively at warm conditions; these accessions maintained significantly higher rates of A than 'LA Purple' after 15 days of chilling, with rates of 13.3 μmol m^{-2} s^{-1} (49.1% decrease), 12.0 μmol m^{-2} s^{-1} (53.1% decrease), and 11.3 μmol m^{-2} s^{-1} (55.8% decrease), respectively; and significantly higher rates of A than 'LA Purple' upon return to 25° C following the 15-day chilling treatment, with rates of 18.5 μmol m^{-2} s^{-1} (28.8% decrease), 23.1 μmol m^{-2} s^{-1} (9.9% decrease), and 20.3 μmol m^{-2} s^{-1} (20.4% decrease), respectively; in comparison to 'LA Purple', which maintained average rates of A at 28.6 μmol m^{-2} s^{-1} at warm conditions, 2.0 μmol m^{-2} s^{-1} (92.6% decrease) after 15 days of chilling, and 3.7 μmol m^{-2} s^{-1} (85.7% decrease) at return to warm conditions. As shown in Fig. 12G, the aforementioned accessions HB07-3329-5 and HB07-3073-6 also showed significantly higher ϕ_{PSII} than 'LA Purple' after 5 days of chilling, with ϕ_{PSII} values of 0.131 (43.9% decrease) and 0.143 (41.1% decrease) compared to 0.033 (85.9% decrease) in 'LA Purple'. No accessions showed higher ϕ_{PSII} than 'LA Purple' after 15 days of chilling (Fig. 12F), and accessions HB07-3023-1, HB07-3043-3, and HB07-3114-9 showed higher ϕ_{PSII} than 'LA Purple' at return to warm conditions (Fig. 12H). All energycane accessions showed similar stomatal conductance of water vapor (g_s, Fig. 13A-G) and increases in the ratio of intercellular-to-atmospheric CO_2 (C_i/C_a, Fig. 14A-G) to 'LA Purple' throughout the 15-day chilling treatment, while energycane accession HB07-3452-4 showed significantly higher g_s than 'LA Purple' at return to warm conditions (Fig. 13H-I), with a 17.2% increase compared to the 86.4% decrease shown by 'LA Purple', and accession HB07-3452-4 showed significantly higher C_i/C_a than 'LA Purple' at return to warm conditions (Fig. 14H-I), with a 27.8% increase compared to the 19.1% decrease shown by 'LA Purple'.

DISCUSSION

In this experiment we identified three energycane hybrids with higher photosynthetic chilling tolerance than 'LA Purple' over a 15-day period of exposure to chilling temperatures of 15°/10° C in controlled environment chambers. This was an early-generation comparison of the photosynthetic chilling tolerance of nineteen energycane hybrids with the sugarcane variety 'LA Purple' which has been previously identified to experience sizable reductions of photosynthesis in exposure to chilling temperatures (Glowacka, *et al*., 2014). Accessions HB07-3452-4, HB07-

3073-6, and HB07-3329-5 experienced lower reductions in A than 'LA Purple' between 5-15 days of chilling, and showed the highest recovery of A among all genotypes as well as 'LA Purple' at re-elevation of temperature to 25° C. Genotypes HB07-3452-4, HB07-3073-6, and HB07-3329-5 suffered reductions in A of 53.1%, 55.8%, and 46.5%, respectively on the 15th day of chilling, compared to a sizable 79.3% reduction in LA Purple. In addition these three accessions showed the highest rates of recovery at re-elevation of temperatures to 25° C, suffering minimal reductions in A (9.9%, 20.4%, and 28.8%, respectively) while 'LA Purple' suffered a 63.1% reduction in A. The impressive tolerance for prolonged chilling in the genotypes discussed above makes them attractive as possible genotypes for cultivation for bio-energy purposes in areas that are exposed to periods of chilling during the growing season, as well as possible materials for ongoing breeding efforts for chilling tolerance.

When attempting breed for improvements in a crop's resistance to abiotic stress it is also fundamentally important to ensure that the performance of hybrids under favorable conditions is not diminished. And indeed, genotypes HB07-3452-4, HB07-3073-6, and HB07-3329-5 maintained comparable high levels of A to those measured in 'LA Purple' at 25°/20° C. Interestingly, although these three genotypes managed to maintain significantly higher A after prolonged exposure to chilling temperatures and at re-elevation of temperatures, they did not maintain higher levels of ϕ_{PSII} from 'LA purple' either at 15° C or at re-elevation of temperatures to 25° C. Although the physiological basis of C4 chilling tolerance in sugarcane remains critically under-explored, parallels can be inferred from the closely related genera *Miscanthus* and *Zea*. Is it possible that regulation of PPDK and rubisco contents (Du *et al*., 1999b; Naidu, *et al*., 2004; Wang *et al*., 2008) account for the retention in A in genotypes HB07-3452-4, HB07-3073-6, and HB07-3329-5? Additionally, is it possible that failure to regulate thylakoid proteins (Fryer et al., 1995; Spence *et al*. 2014), accumulate zeaxanthin (Farage *et al*. 2006), or increase expression of reactive oxygen-scavenging enzymes (Jahnke *et al*., 1991; Fryer *at al*., 1998; Ort and Baker, 2002) in exposure to chilling temperatures result in the lack of recovery in ϕ_{PSII}?

These chilling-tolerant genotypes did not share any common parental lines: genotype HB07-3452-4 was derived from a hybridization involving the released energycane variety L79-1002 and an F1 hybrid with *S. spontaneum* ancestry, and accessions HB07-3073-6 and HB07-3329-5 were derived from hybridizations between commercial breeding clones and *S. spontaneum* parents (Table 1). Unfortunately, the comparison of these genotypes with their respective parental lines was not possible in this study. As such, they were compared to the commercial variety 'LA Purple' due to its known susceptibility to chilling damage (Glowacka, *et al*., 2014)

At present, *S. spontaneum* germplasm is the primary source of genes for improvement of sugarcane tolerance of biotic/abiotic stresses, and populations of *S. spontaneum* originating from environments ranging from Honshu, Japan to tropical Southeast Asia are widely used in breeding for genetic gains in this crop. Brandes (1940) found that vegetative stems of some *S. spontaneum* from Turkestan managed to survive 18 days below freezing temperatures in January and re-sprouted new shoots in April, and Hale *et al*. (2014) identified four accessions of *S. spontaneum* with higher ratoon freezing tolerance than the freezing-tolerant sugarcane variety 'HoCP 96-540'. Additionally, Friesen *et al.* (2014) found that an energycane F1 hybrid ('Ho 02-113') of the popular sugarcane variety 'LCP 85-384' and *S. spontaneum* from the Himalayan foothills of northern India managed to recover 63% of its original CO_2 assimilation rate upon return to 25 °C/20 °C following a 6-day chilling treatment of 12°C/5°C. And Du *et al.* (1999a) showed that a warm-grown (30° C) complex *S. officinarum* x *S. spontaneum* x *S. barberi* hybrid managed to retain nearly 74% of its photosynthetic rate after 52 hours transfer from 30° C to 10° C, compared to *S. officinarum* cv. Badira, which only retained 22% of its photosynthetic rate. As such, the acute chilling sensitivity of *S. officinarum* may be attenuated through transfer of genes conferring chilling-tolerance from chilling-tolerant lines of *S. spontaneum*. The results presented in this study add to a growing body of works that suggests that the use of *S. spontaneum* parental lines may generate genotypes with superior resistance to chilling temperatures.

In conclusion, the phenotypic evaluation of energycane lines derived from the basic USDA-ARS breeding program in Houma, Louisiana proved to be a fruitful endeavor, supporting the hypothesis that genotypes with superior photosynthetic chilling tolerance can produced by crossing elite sugarcane lines with *S. spontaneum* germplasm. Of note are genotypes HB07-3452-4, HB07-3073-6, and HB07-3329-5 which matched and out-performed the majority of other genotypes in their capacity to maintain high photosynthetic rates even after 15 days of exposure to 15° C. These genotypes may prove to be useful in future breeding efforts for sugarcane chilling tolerance, which must account for the regulation of enzymes involved in the C4 cycle, enzymes involved in maintenance of healthy PSII complexes, enzymes involved in the xanthaphyll cycle, enzymes involved in the water-water cycle, and reactive oxygen-scavenging enzymes in response to chilling.

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TABLES & FIGURES

Table 2 Sugarcane parental lines used in the hybridization of each genotype. Genotypes, female, and male parents are described by their generation as: released energycane variety (REC), commercial breeding clone (CBC), wild *Saccharum spontaneum* (*Ss*), wild *Erianthus arundinaceous* (*Ea*), F1 hybrids (F1), hybrids derived from crosses involving F1 parents (F1'), 1st backcrossing generation (BC1), 2nd backcrossing generation (BC2), and polycross (POLY). The naming convention of genotypes indicates "HB" for the "Houma Basic" breeding scheme, "07" indicates the year in which the crosses were made, the second number-set that starts with "3000" indicates the specific cross from which the material was derived, and the last hyphenated number-set indicates the specific seedling number from which each genotype was clonally propagated (i.e. HB07-2023-1 and HB07-2023-2 are full sibs).

Genotype	Current generation	Female parent identifier	Female generation	Male parent identifier	Male generation
HB07-3022-2	F1'	US06-9022	F1	US05-9017	F1
HB07-3023-1	F1'	MPTH97-194	Ss	US05-9017	F1
HB07-3023-2	F1'	MPTH97-194	Ss	US05-9017	F1
HB07-3041-1	POLY	MPTH97-194	Ss	07EriPoly	Ea
HB07-3041-3	POLY	MPTH97-194	Ss	07EriPoly	Ea
HB07-3042-2	POLY	MPTH97-221	Ss	07EriPoly	Ea
HB07-3043-2	POLY	MPTH98-283	Ss	07EriPoly	Ea
HB07-3043-3	POLY	MPTH98-283	Ss	07EriPoly	Ea
HB07-3043-8	POLY	MPTH98-283	Ss	07EriPoly	Ea
HB07-3045-11	F1	HoCP04-838	REC	MPTH97-216	Ss
HB07-3045-12	F1	HoCP04-838	REC	MPTH97-216	Ss
HB07-3045-2	F1	HoCP04-838	REC	MPTH97-216	Ss
HB07-3073-2	F1	HoCP04-809	CBC	MPTH97-204	Ss
HB07-3073-6	F1	HoCP04-809	CBC	MPTH97-204	Ss
HB07-3114-5	F1'	MPTH97-194	Ss	MPTH97-204	Ss
HB07-3114-8	F1'	MPTH97-194	Ss	MPTH97-204	Ss
HB07-3114-9	F1'	MPTH97-194	Ss	MPTH97-204	Ss
HB07-3261-8	BC2	US06-9001	BC1	L99-226	REC
HB07-3314-3	F1'	MPTH97-194	Ss	MPTH97-204	Ss
HB07-3329-1	F1	HoCP01-517	CBC	MPTH97-209	Ss
HB07-3329-3	F1	HoCP01-517	CBC	MPTH97-209	Ss
HB07-3329-5	F1	HoCP01-517	CBC	MPTH97-209	Ss
HB07-3329-8	F1	HoCP01-517	CBC	MPTH97-209	Ss
HB07-3452-3	F1'	L79-1002	REC	US02-144	F1
HB07-3452-4	F1'	L79-1002	REC	US02-144	F1

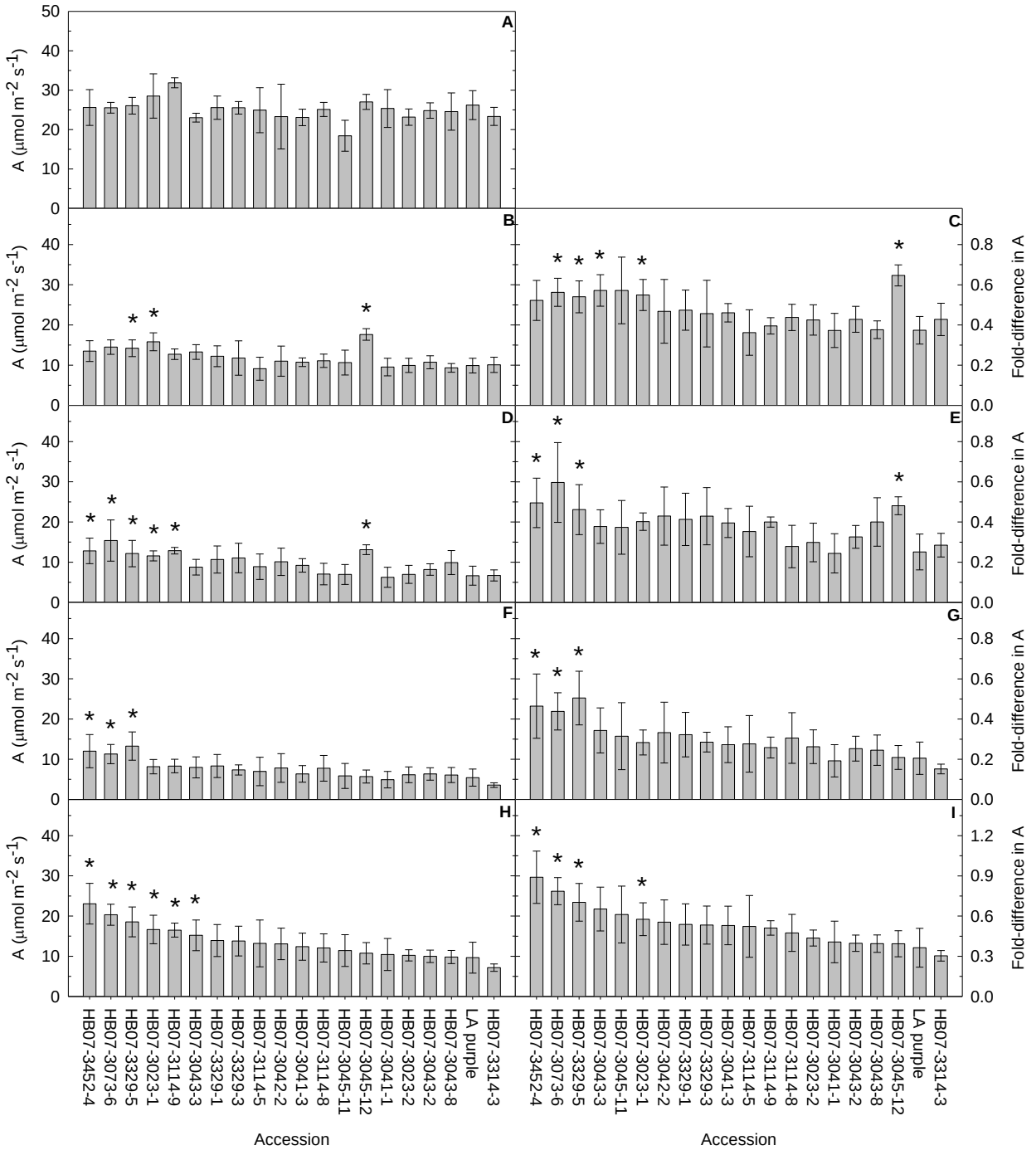

Fig. 11 The effect of a 15-day chilling treatment on leaf carbon assimilation rates under saturating light (1500 µmol m^{-2} s^{-1}) for each sugarcane genotype and LA Purple. The left column depicts means (n=3 to 5) and standard error of carbon assimilation rates, and the right graph column depicts means (n=3 to 5) and standard error of the rate of carbon assimilation relative to the measurements at 25° C before initiation of the chilling treatment. Carbon assimilation rates (A) at 25° C before initiation of chilling treatment, (B & C) at 15° C during the first day of chilling treatment, (D & E) at 15° C 5 days after initiation of chilling treatment, (F & G) at 15° C 15 days after initiation of chilling treatment, and (H & I) at 25° C on the first day after re-elevation of temperatures. In all panels per each graph column, genotypes are ordered by values obtained during the last day of measurements at 25° C (panels H and I, respectively). Plants were grown at 25° /20° C day/night and 15° /10° C day/night , and a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity.

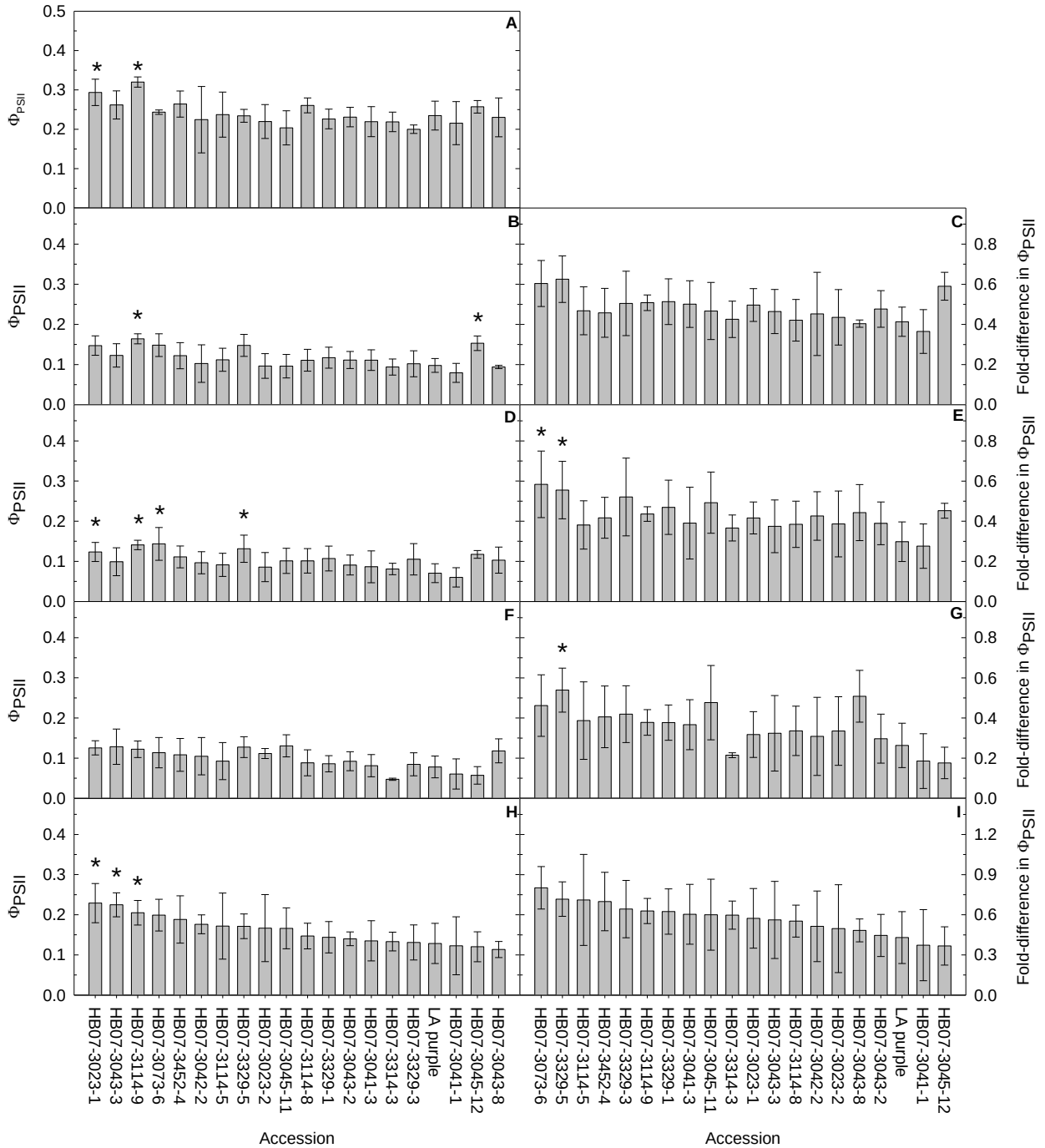

Fig. 12 The effect of a 15-day chilling treatment on quantum yield of photosystem II (ϕ_{PSII}) measured at 1500 µmol photons m^{-2} s^{-1} for each sugarcane genotype and LA Purple. The left column (1) depicts means (n=3 to 5) and standard error of ϕ_{PSII}, and the right graph column (2) depicts means (n=3 to 5) and standard error of ϕ_{PSII} relative to the measurements at 25° C before initiation of the chilling treatment. ϕ_{PSII} values (A) at 25° C before initiation of chilling treatment, (B & C) at 15° C during the first day of chilling treatment, (D & E) at 15° C 5 days after initiation of chilling treatment, (F & G) at 15° C 15 days after initiation of chilling treatment, and (H & I) at 25° C on the first day after re-elevation of temperatures. In all panels per each graph column, genotypes are ordered by values obtained during the last day of measurements at 25° C (panels H and I, respectively). Plants were grown at 25° /20° C day/night and 15° /10° C day/night , and a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity.

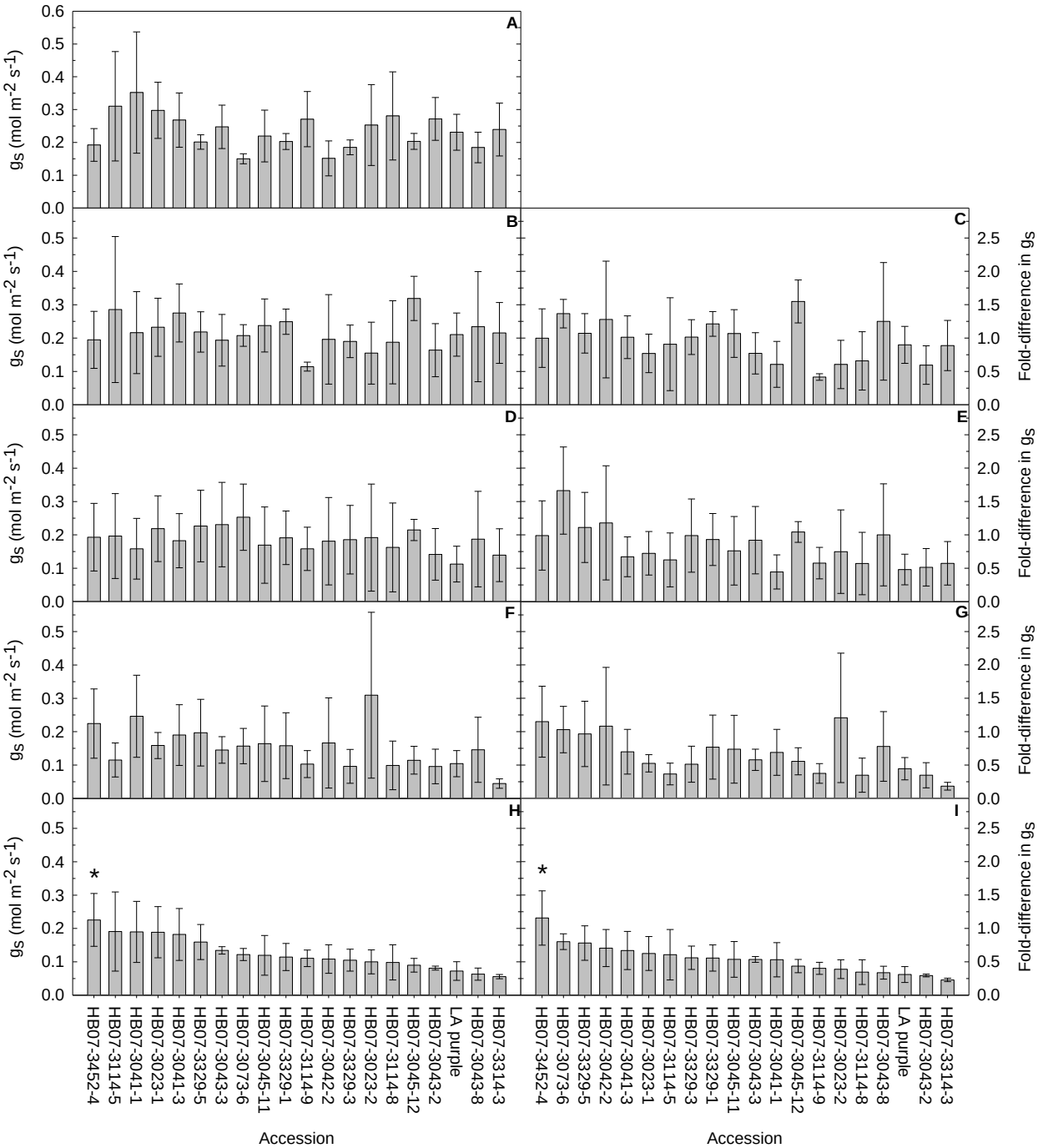

Fig. 13 The effect of a 15-day chilling treatment on stomatal conductance of water vapor (g_s) measured at 1500 µmol photons m^{-2} s^{-1} for each sugarcane genotype and LA Purple. The left column depicts means (n=3 to 5) and standard error of g_s, and the right graph column depicts means (n=3 to 5) and standard error of g_s relative to the measurements at 25° C before initiation of the chilling treatment. Stomatal conductance values (A) at 25° C before initiation of chilling treatment, (B & C) at 15° C during the first day of chilling treatment, (D & E) at 15° C 5 days after initiation of chilling treatment, (F & G) at 15° C 15 days after initiation of chilling treatment, and (H & I) at 25° C on the first day after re-elevation of temperatures. In all panels per each graph column, genotypes are ordered by values obtained during the last day of measurements at 25° C (panels H and I, respectively). Plants were grown at 25° /20° C day/night and 15° /10° C day/night , and a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity.

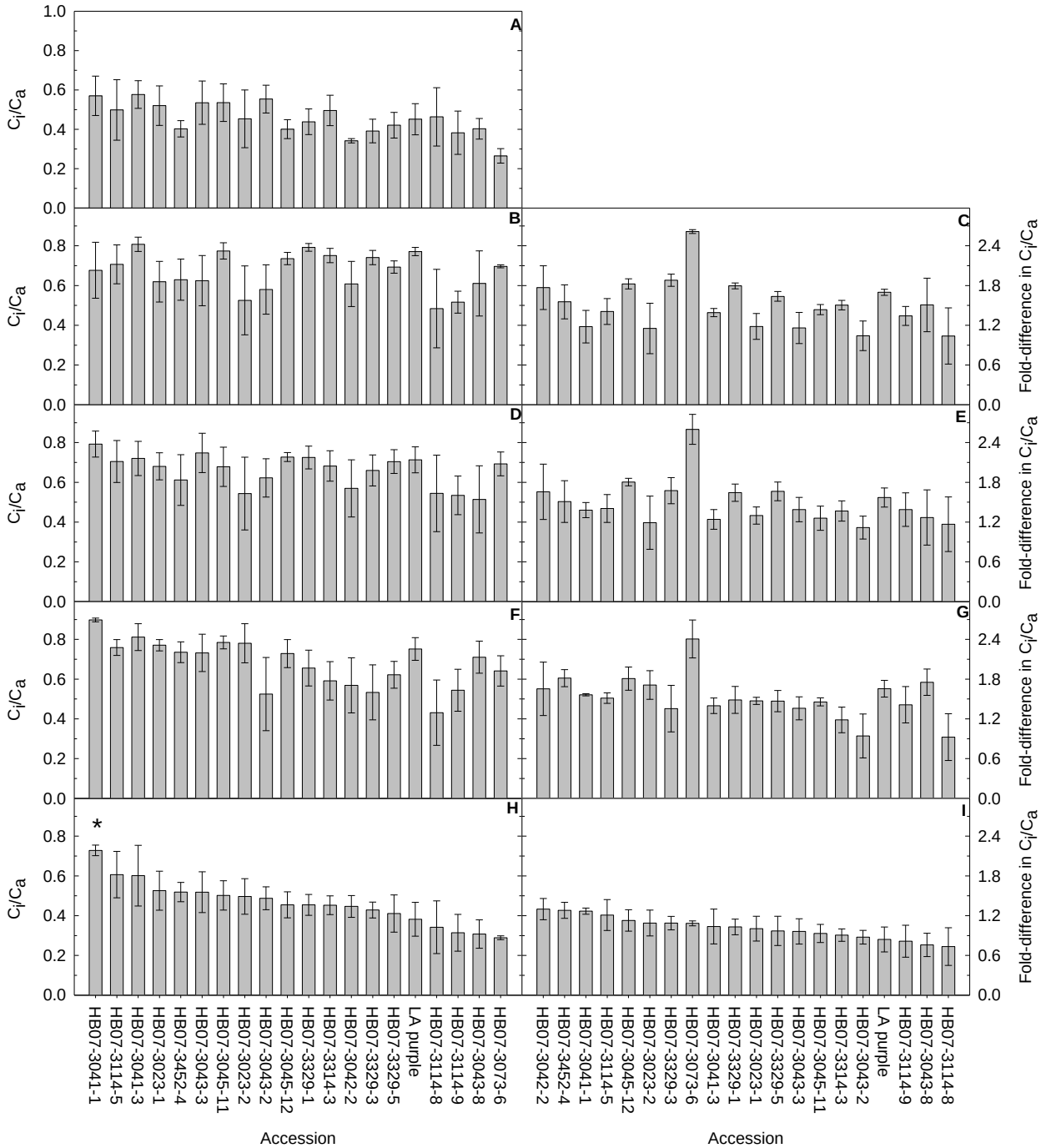

Fig. 14 The effect of a 15-day chilling treatment on the ratio of intercellular to atmospheric CO_2 (C_i/C_a) measured at 1500 µmol photons m^{-2} s^{-1} for each sugarcane genotype and LA Purple. The left column depicts means (n=3 to 5) and standard error of C_i/C_a, and the right graph column depicts means (n=3 to 5) and standard error of C_i/C_a relative to the measurements at 25° C before initiation of the chilling treatment. Ratio of intercellular to atmospheric CO_2 (A) at 25° C before initiation of chilling treatment, (B & C) at 15° C during the first day of chilling treatment, (D & E) at 15° C 5 days after initiation of chilling treatment, (F & G) at 15° C 15 days after initiation of chilling treatment, and (H & I) at 25° C on the first day after re-elevation of temperatures. In all panels per each graph column, genotypes are ordered by values obtained during the last day of measurements at 25° C (panels H and I, respectively). Plants were grown at 25° /20° C day/night and 15° /10° C day/night , and a 14 h /10 h day/night dark cycle under 800 µmol photons m^{-2} s^{-1} illumination, and 75% relative humidity.

CHAPTER 4

CONCLUDING REMARKS

The research presented in this thesis shows that opportunity exists in wild germplasm material for the improvement of severe chilling tolerance in *Miscanthus x giganteus*, and that superior chilling tolerance can be generated in energycane through specific crosses between superior sugarcane varieties and *S. spontaneum*. In *Miscanthus* and energycane, severe chilling (<12° C) and moderate chilling (>14° C), respectively stifled the capacity for recovery under chilling conditions. Maintenance of photosynthetic rates under chilling conditions and recovery at re-elevated temperatures are suggested here to be a function of chilling-induced regulation of specific enzymes involved in the C4 cycle, maintenance of thylakoid membrane proteins, light-energy dissipation strategies, generation of alternate electron sinks, and reactive oxygen scavenging as observed in C4 crops in response to chilling temperatures (Jahnke *et al*., 1991; Ort and Baker, 2002; Naidu and Long, 2003; Naidu *et al.*, 2004; Farage *et al.*, 2006; Fryer *at al*., 1998; Wang *et al.*, 2008; Dohleman and Long, 2009; Long and Spence, 2013; Spence *et al.*, 2014).

Miscanthus x giganteus is a leading feedstock candidate for biomass production in temperate climates, and potential enhancements to its productivity and length of growing season only serve to augment the argument for its cultivation for lignocellulosic biomass production over C3 crops in areas where chilling temperatures limit the yields of C4 crops. Although the physiological response and gene expression patterns of *Mxg*'s chilling tolerance of mild chilling temperatures are well characterized (Beale *et al*., 1996; Naidu and Long, 2003; Naidu *et al.*, 2004; Farage *et al.*, 2006; Wang *et al.*, 2008; Long and Spence, 2013; Spence *et al.*, 2014; Glowacka et al., 2015), the physiological response and gene expression patterns of *Mxg* to severe chilling temperatures has yet to receive substantial empirical attention. The results presented in chapter 2 add to the growing body of work on the chilling response of *Mxg* at severe chilling temperatures (Farage *et al*., 2006; Purdy *et al*., 2013; Glowacka *et al*. 2014; Friesen *et al*., 2014), and shows that within previously untapped genetic resources in wild germplasm there is room for improvement of the already exceptional chilling tolerance of *Mxg.* In this chapter we evaluated the chilling tolerance of 91 Siberian *Miscanthus sacchariflorus* accessions, and highlighted accession RU2012-114 for its impressive tolerance of severe chilling temperatures and tolerance of overnight frosts.

Sugarcane is another highly productive crop in tropical and subtropical climates, but its high susceptibility to chilling damage and reductions in yields in response to chilling temperatures have limited its cultivation in N. America (Verret and Das, 1927;Clements, 1980; Grantz, 1989). Energycane, much like *Mxg*, is regarded as a leading feedstock candidate for lignocellulosic biomass production in warm temperate climates (Bransby *et a*l., 2010), and enhancements to the chilling tolerance of this crop can serve to expand the bounds and yields of energycane production in the United States (Knoll *et al*., 2013). The results presented in chapter 3 highlight three energycane hybrids that were generated from the USDA-ARS basic sugarcane breeding program with superior photosynthetic chilling capacities (HB07-3452-4, HB07-3073-6, HB07-3329-3). This work shows that gains in photosynthetic chilling tolerance of energycane are possible through specific crosses between elite varieties and wild *S. spontaneum* germplasm and it adds to the shallow pool of empirical evaluations of photosynthetic chilling response in sugarcane (Grantz 1989; Du *et al*., 1999; Friesen *et al*., 2014).

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